Mutational resistance to linezolid and other anti-gram-positive antibiotics in staphylococci

Thesis

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Mutational resistance to linezolid and other anti-gram-positive antibiotics in staphylococci

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) are endemic in UK hospitals. Treatment options for the infections caused by these multiresistant strains include vancomycin, teicoplanin and linezolid. Mutational resistance to these antibiotics, although rare in the clinic, has been detected. Resistance to linezolid requires at least one chromosomal mutation followed by subsequent internal recombination. The hypothesis tested was that resistance to linezolid or glycopeptides could emerge in hypermutable strains and that hypermutability could be co-selected with resistance to linezolid or glycopeptides. Accordingly, this work studied linezolid and teicoplanin-resistant clinical isolates and laboratory-selected linezolid-resistant *S. aureus* mutants for evidence of hypermutability.

Mutation frequencies to a range of antibiotics were determined to evaluate the occurrence of hypermutability. Linezolid-resistant mutants were selected by serial passage in the presence of increasing concentrations of linezolid. Mutations conferring linezolid resistance were characterized by PCR-RFLP and sequencing. Pyrosequencing and hybridization were used to detect and quantify six mutations known to confer linezolid resistance.

Pre-existing hypermutability increased the ability of a laboratory strain to generate linezolid resistant mutants. However, few clinical linezolid or teicoplanin-resistant isolates or mutants were found to be hypermutable, indicating that stable hypermutability is not a prerequisite for the emergence of these resistances. Likewise, no laboratory-selected linezolid-resistant mutants were hypermutable, demonstrating the lack of co-selection of linezolid resistance with hypermutability. Most linezolid-resistant laboratory-selected mutants were unstable. There was a direct correlation between the number of mutated 23S rRNA genes and linezolid MIC in clinical and laboratory mutants, although analysis was complicated by the fact that the 23S rRNA gene copy number was variable among the laboratory mutants. Cross-resistance between linezolid and chloramphenicol was detected in some laboratory mutants.

In conclusion, there was no evidence for the co-selection of hypermutability and linezolid or teicoplanin resistance, and hypermutability was not found to be a prerequisite for the emergence of linezolid resistance.

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"I've managed to climb a mountain that was much too big for me" Anon 2003

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Abbreviations

°C	Degrees Celsius
µg	Microgram(s)
µl	Microlitre(s)
A	Adenine
ARMRL	Antibiotic Resistance Monitoring and Reference Laboratory
ATCC	American Type Culture Collection
ATP	Adenosine tri-phosphate
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
bp	Base pairs
BSAC	British Society for Antimicrobial Chemotherapy
C	Cytosine
CA	Columbia agar
CA-MRSA	Community acquired MRSA
CBA	Columbia blood agar
CF	Cystic fibrosis
cfu/ml	Colony forming units per ml
Chlor	Chloramphenicol
Cip	Ciprofloxacin
Clin	Clindamycin
CNS	Coagulase-negative staphylococci
cSSSI	Complicated skin and skin structure infections
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
DHFR	Dihydrofolate reductase
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
EARSS	European Antimicrobial Resistance Surveillance System
EMRSA	Epidemic MRSA

Cont.

Abbreviations (cont.)

Ery	Erythromycin
FDA	Food and Drug Administration (USA)
Fus	Fusidic acid
GES	Guanidinium thiocyanate
g	Gram(s)
G	Guanine
G-6-P	Glucose-6-phosphate
GISA	Glycopeptide-intermediate *S. aureus*
GRE	Glycopeptide-resistant enterococci
H. halobium	*Halobacterium halobium*
HARMONY	Harmonisation of Antibiotic Resistant ways of measurement, Methods of typing Organisms and using these and other tools to increase the effectiveness of Nosocomial infection control
H. influenzae	*Haemophilus influenzae*
HPA	Health Protection Agency
IF	Initiation factor
ILP	Intergenic spacer length polymorphism
ISO	IsoSensitest
Kb	Kilobases
L	Litre(s)
Lin	Linezolid
M	Molar
mBar	Millibar
mg/L	Milligrams per litre
MHA	Muller-Hinton agar
MIC	Minimum inhibitory concentration
min	Minute(s)
ml	Millilitre
MLS_B	Macrolides, lincosamides, streptogramin B
MLST	Multi-locus sequence typing
mm	Millimeter(s)
mM	Millimolar
MMR	Mismatch repair
mRNA	Messenger RNA
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-susceptible *S. aureus*

Cont.

Abbreviations (cont.)

NA	Nutrient agar
N/A	Not applicable
NaCl	Sodium chloride
NB	Nutrient broth
NCTC	National Collection of Type Cultures
nm	Nanometer(s)
Oxa	Oxacillin
P. aeruginosa	*Pseudomonas aeruginosa*
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Hydrogen ion potential
PPi	Pyrophosphate
PROTEKT	Prospective resistant organism tracking and epidemiology for the ketolide telithromycin
PVL	Panton-Valentine leukocidin
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
Rif	Rifampicin
RNA	Riboxynucleic acid
rpm	Revolutions per minute
rrn	Ribosomal RNA operon
rRNA	Ribosomal RNA
S	Svedberg unit (sedimentation coefficient)
S. aureus	*Staphylococcus aureus*
SD	Standard deviation
S. epidermidis	*Staphylococcus epidermidis*
S. haemolyticicus	*Staphylococcus haemolyticicus*
S. pneumoniae	*Streptococcus pneumoniae*
S. sciuri	*Staphylococcus sciuri*
SCC*mec*	Staphylococcal cassette chromosome *mec*
sec	Second(s)
T	Thymine
TBE	Tris-borate EDTA
tDNA	Transfer DNA

Cont.

Abbreviations (cont.)

TE	Tris EDTA
Teic	Teicoplanin
tRNA	Transfer RNA
UK	United Kingdom
USA	United States of America
UTI	Urinary tract infection
UV	Ultraviolet
Vanc	Vancomycin
VISA	Vancomycin-intermediate *S. aureus*
V	Volts
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *S. aureus*
WT	Wild-type
x g	Gravitational force

1 Introduction

1.1 Staphylococcus

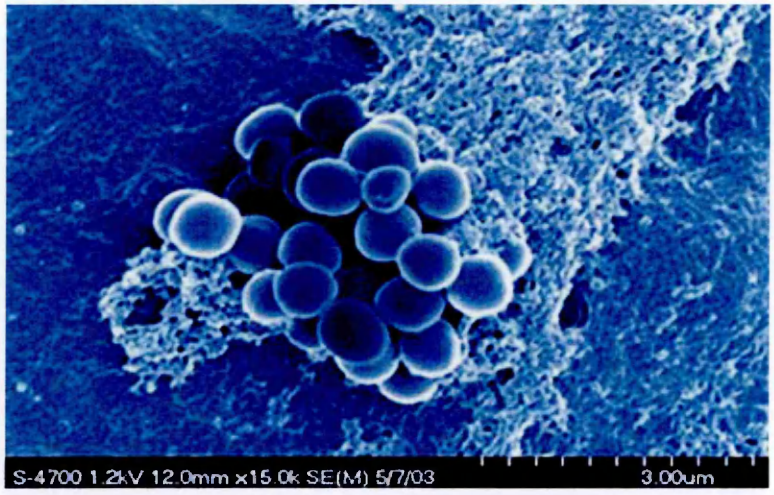
1.1.1 Description

Staphylococci were first described by Sir Alexander Ogsten in 1880, after he observed them in pus from human abscesses. He named the organisms after the Greek words for grape (staphyle) and berry (kokkos) due to their appearance (Figure 1). In 1884 staphylococci were isolated from a wound and grown for the first time in pure culture by Rosenbach (Baird-Parker, 1990).

Staphylococci are non-motile, gram-positive, catalase-positive, chemoorganotrophic, facultative anaerobes that are non-spore forming. Staphylococcal colony colour varies with species, ranging from opaque to orange (Holt, 1994). The natural habitat of staphylococci is the skin and mucous membranes of warm-blooded animals (Kloos, 1990). Problems associated with staphylococcal species arise when the natural, cutaneous barrier between bacterium and host is breached through trauma, surgery or indwelling surgical devices (Kloos, 1980).

In terms of classification, staphylococci belong to the Order Bacillales, Bacterial family Staphylococcaceae, genus *Staphylococcus* (http://141.150.157.80/bergeysoutline/outline/bergeys outline _5_2004.pdf). As of January 2006, there are 40 species and 24 sub-species within the genus *Staphylococcus* (http://www.bacterio.cict.fr/s/staphylococcus.html). The best-known and most clinically important of these is *Staphylococcus aureus* (*S. aureus*) (Holt, 1994). The genus *Staphylococcus* is traditionally divided into two broad categories based on the ability of some species to coagulate blood plasma. Coagulase-positive species, include *S. aureus* (principally), *Staphylococcus intermedius* and *Staphylococcus delphini*; coagulase-negative species (CNS) includes *Staphylococcus epidermidis* (*S. epidermidis*) (Kloos, 1990).

Figure 1. Scanning electron microscopy image of *S. aureus* on epithelial cells. (From http://www.microbiology.med.umn.edu/microbiology/seminars/mmpc.html)



1.1.2 Delineation of species

1.1.2.1 Phenotypic identification

The coagulase test was introduced in 1925 by Von Dáranyi and is still an important tool to discriminate *S. aureus* from less pathogenic CNS species (Kloos, 1980). Commercial phenotypic identification systems, such as API Staph and ID 32 Staph (bióMerieux), provide identification to species level within 24 hours. Nevertheless, they are less than fully reliable for some more obscure species, such as *Staphylococcus vitulinus*, which are not included in the reference databases of these systems, possibly resulting in mis-identification (Stepanovic *et al.,* 2005). Automated identification systems, such as the Vitek system (bioMérieux) and the BD (Becton-Dickinson) Phoenix system, are a more recent development and also perform susceptibility testing. Reports on the accuracy of these systems are conflicting (Heikens *et al.,* 2005, Ligozzi *et al.,* 2002). Ultimately, phenotypic characterizations are marred by the fact that they depend on the expression of metabolic or morphological features which may vary between strains (Becker *et al.,* 2004). With the introduction of molecular techniques, genotypic identification has proved superior.

1.1.2.2 Genotypic identification

Deoxyribonucleotide genetic divergence (DNA-DNA homology) is now the 'gold standard' for the definition of new staphylococcal species and subspecies, with dissimilar species having relative hybridization values of less than 70% (Kloos, 1980). Nevertheless, this method is lengthy and more rapid methods have been introduced. Amplification and partial sequencing of gene targets has been assessed as a technique for staphylococcal species identification. Within a genus, highly-conserved regions of an essential gene are amplified, followed by partial DNA sequencing (Heikens *et al.,* 2005). In the search for a suitable molecular target, a variety of genes have been assessed, e.g., 16S rRNA (ribosomal ribonucleic acid) (Heikens *et al.,* 2005), superoxidase dismutase A (*sodA*) (Poyart *et al.,* 2001), RNA polymerase B (*rpoB*) (Drancourt and Raoult, 2002), elongation factor Tu (*tuf*) (Martineau *et al.,* 2001) and heatshock protein 60 (*hsp60*) (Kwok *et al.,* 1999). In a comparison of these techniques, Mellman *et al.*, (2006) found that using *rpoB* as a

molecular target provided the greatest differentiation between species, enabling successful identification of 38 staphylococcal species and subspecies. Similarly, Khamis *et al.*, (2005) found partial *rpoB* sequencing a simple and efficient means of identifying species of *Corynebacterium*. However, the drawback to using *rpoB* as a target gene is the generally poor availability of published sequences, as compared with 16S rRNA (Mellmann *et al.*, 2006).

Ribotyping is a technique that allows subtype differentiation beyond species and subspecies level. It is utilised for both species identification and also as an epidemiological tool for strain typing. A fingerprint, based on a restriction pattern, is produced by hybridizing digested chromosomal DNA with a complementary DNA (cDNA) probe derived from rRNA. Differences within rRNA genes and their flanking regions result in fingerprint variations. Izard *et al.*, (1992) found ribotyping of little use for differentiating strains of coagulase-negative staphylococci and concluded that similarities in ribotypes between species interfered with interspecies variation. In this instance, the epidemiological value of ribotyping outweighs its capacity as a taxonomic tool.

PCR (polymerase chain reaction) -amplified tDNA (transfer DNA) intergenic spacer length polymorphism (tDNA-ILP) was evaluated as a tool for species identification by Welsh *et al.*, (1992). Conserved tDNA genes are used as a basis for designing genus-specific primers to amplify variable spacer regions. The resulting PCR products are species-specific in size. Maes *et al.*, (1997) used tDNA-ILP to identify, successfully, 15 staphylococcal species with 99% accuracy, whilst Welsh *et al.*, (1992) proposed that intergenic spacer regions between 16S and 23S or 23S and 5S rRNA genes could also be used as a means of identifying staphylococci to species level, a technique Mendoza *et al.*, (1998) and Couto *et al.*, (2001) found to be successful. To enable greater discrimination between species it is possible to use restriction fragment length polymorphism (RFLP) analysis of the intergenic 16-23S rRNA PCR product (Mendoza *et al.*, 1998, Sudagidan *et al.*, 2005). A comparison of DNA-ILP identification techniques revealed both tDNA-ILP and 16-23S rRNA-ILP RFLP to be potentially important techniques for quick and precise staphylococcal species identification (Lee and Park, 2001). However, the lack of variation within species means the identification of subspecies is difficult (Mendoza *et al.*, 1998).

PCR still remains the quickest and most economical technique for identification of the most common staphylococcal species. Gribaldo *et al.*, (1997) designed a PCR assay, based on three

variable regions in the 16S rRNA gene, to identify eight common staphylococcal species. More recently, Sabat *et al.*, (2004) described a single-tube multiplex-PCR for the discrimination of eleven *Staphylococcus* species, based on the amplification of regions of genes encoding metalloproteinases from the thermolysin family, the results of which were in agreement with partial sequencing of 16S rRNA and *rpoB*.

1.1.3 Clinical role of staphylococci

Staphylococci, particularly *S. aureus*, are common causes of bloodstream and other infections in hospitalised patients. CNS live naturally on the skin and mucous membranes of humans and are therefore often found in clinical specimens (O'Gara and Humphreys, 2001, von Eiff *et al.,* 2001). Distinguishing clinically significant, pathogenic CNS from contaminants is therefore difficult and it is often thought that the isolation of CNS species from blood samples is due to contamination when the specimen is taken (O'Gara and Humphreys, 2001, von Eiff *et al.,* 2002). Nevertheless, CNS are by far the most common causes of bacteraemia related to indwelling devices (Huebner and Goldmann, 1999).

Outbreaks of both *S. aureus* and CNS infections in hospitals are often the result of nosocomial transmission of clones, which are often multi-resistant (see section 1.2) (Boyce *et al.,* 1993, Huebner and Goldmann, 1999). To distinguish these clones from sporadic isolates, molecular epidemiological typing techniques are required (Aber and Mackel, 1981). These techniques rely on the fact that epidemiologically-related isolates, being descendants of a common ancestor, have relatively similar genomic fingerprints (Maslow *et al.,* 1993, Shopsin and Kreiswirth, 2001). As a result of typing, measures can be put into place to interrupt and control strain transmission (Weller, 2000). In the face of increasing MRSA bacteraemia rates, political pressure and media interest, typing techniques have become more important than ever in the battle to control the spread of MRSA.

1.1.4 Methods for typing staphylococci

Typing systems need to be broadly applicable, reproducible, discriminatory, based on a stable property, inexpensive, readily available, standardized and field-tested (Aber and Mackel, 1981). Very few meet all of these criteria.

Bacterial phage typing has been the mainstay of *S. aureus* typing techniques for many years. The realization that *S. aureus* contained phages which could lyse some bacteria of the same species led to the development of a technique enabling strain discrimination based on their phage susceptibility (Wentworth, 1963). An agar plate, covered with the test organism, is inoculated with 23 standard phages and the degree of lysis produced by each phage is recorded, resulting in the production of a phage pattern or type (Weller, 2000, Wentworth, 1963). The procedure relies on the propagation of phage stocks and has low reproducibility (Weller, 2000). A further disadvantage is the non-typability of many strains (Weller, 2000). Bannerman *et al.*, (1995) and Schlichting *et al.*, (1993) found 32% and 30% of strains, respectively, were not typeable by phage analysis. Owing to these disadvantages, phage typing is being replaced by more discriminatory techniques.

Adaptation of a pulsed-field gel electrophoresis (PFGE) procedure, first utilized in yeasts, has led to the production of one of the most widely used strain discrimination techniques (Chu *et al.*, 1986). This technique involves the interpretation of profiles that arise as a result of the macro-restriction of DNA with an enzyme that cuts infrequently, producing few, large DNA fragments (50-700 Kb) (Tenover *et al.*, 1994). The patterns produced show distinct, well-separated fragments and are highly reproducible (Maslow *et al.*, 1993). All isolates are typeable and the discriminatory ability of the technique is high (Tenover *et al.*, 1997). Conversely, the technique is expensive to set up, technically demanding and time-consuming, with the procedure taking four days (Bannerman *et al.*, 1995, Maslow *et al.*, 1993). A further major disadvantage is the lack of standardized interpretative criteria (Tenover *et al.*, 1994, Weller, 2000), although guidelines have been published by the National Centre for Infectious Diseases (USA) (Tenover *et al.*, 1995) and HARMONY (Europe) (http://www.harmony-microbe.net/microtyping.htm). Recent advances in computer programming have assisted with the analysis of PFGE profiles, but inter-laboratory comparison still proves difficult (Cookson *et al.*, 1996, van Belkum *et al.*, 1998). Nevertheless, PFGE proved superior when compared to phage typing (Bannerman *et al.*, 1995), capsular typing (Schlichting *et*

al., 1993), ribotyping (Prevost *et al.,* 1992) and RAPD (Saulnier *et al.,* 1993) and remains the 'gold standard' for defining local outbreaks of *S. aureus* infection.

Recently, *spa* typing has been developed for *S. aureus*. This involves the amplification and subsequent sequencing of the *spa* gene, which encodes for protein A (Koreen *et al.,* 2004). The technique relies on diversity within this gene, arising from the deletion and duplication of 24 bp repetitive units and point mutations (Frenay *et al.,* 1996). The existence of well-conserved regions flanking the *spa* gene allows amplification and sequence typing (Shopsin *et al.,* 1999). Shopsin *et al.,* (1999) and Koreen *et al.,* (2004) evaluated *spa* typing and found it comparable with other techniques such as PFGE and RFLP typing. All isolates were typeable, interpretation is easy, the process is rapid and the technique allows database creation and interlaboratory reproducibility, as shown by Aires-de-Sousa *et al.,* (2006). These advantages probably outweigh the fact that *spa* typing does not have the discriminatory power of PFGE when analysing subtypes (Shopsin *et al.,* 1999).

More recently, multilocus sequence typing (MLST) was proposed as a means for strain differentiation and, additionally, for determining the evolutionary history of clones (Enright *et al.,* 2000, Enright *et al.,* 2002). The technique involves sequencing approximately 450 bp of each of seven housekeeping genes. The sequence for each gene is assigned a distinct allele number and, in combination, the seven alleles make up an allelic profile or sequence type (Enright *et al.,* 2000). Isolates with the same sequence type are assumed to belong to the same clone (Maiden *et al.,* 1998). The method is highly discriminatory and the inter-laboratory exchange of sequence data is straightforward (Enright *et al.,* 2000). As well as using MLST to investigate recent clonal outbreaks, the technology has been used in combination with SCC*mec* (*staphylococcal* cassette chromosome) type (see section 1.2.3) as a population tool to gain insight into the long-term evolutionary history of MRSA clones (Crisostomo *et al.,* 2001, Enright *et al.,* 2002).

1.2 Antibiotic resistance in *Staphylococcus aureus*

1.2.1 Emergence of antibiotic resistance in *S. aureus* (pre-MRSA)

Long before MRSA became a problem, strains of *S. aureus* were already emerging as multi-resistant. The 1940s saw the emergence of *S. aureus* strains resistant to penicillin. This was quickly followed by resistance to tetracycline, erythromycin and streptomycin in the 1950s and 60s. All the time these multi-resistant strains were becoming more prevalent in hospitals and, later on, in the community too. This section discusses the emergence of these early resistance mechanisms in more detail.

1.2.1.1 Penicillin resistance

Penicillin acts by inactivating the penicillin-binding proteins (PBPs) which catalyse the last steps in cell wall synthesis, resulting in reduced cell wall crosslinking. Penicillin was introduced into clinical use in the 1940s, however, resistant strains of *S. aureus* emerged shortly afterwards. Research by Kirby (1944) demonstrated that penicillin-resistant strains of *S. aureus* were able to inactivate penicillin. Further work by Bondi *et al.*, (1945) identified that the enzyme penicillinase was responsible for this inactivation. It has since been elucidated that resistance to penicillin is mediated by the *blaZ* gene which encodes the β-lactamase enzyme (also referred to as penicillinase) (Barber and Rozwadowska-Dowzenko, 1948). The enzyme hydrolyses the β-lactam ring, rendering the antibiotic inactive (Bush, 1988). The *blaZ* gene is usually plasmid-mediated, although chromosomal integration is frequent (Bush, 1989). In 1948 a survey of a UK hospital revealed 59% of *S. aureus* infections were penicillin-resistant (Barber and Rozwadowska-Dowzenko, 1948, Rammelkamp and Maxon, 1942). A survey conducted in the UK in 2004 revealed 90% of *S. aureus* strains isolated from bacteraemias, were resistant to penicillin (http://www.bsacsurv.org/mrsweb /bacteraemia). It is thought that the spread of penicillin-resistant strains could be attributed to the horizontal transfer of plasmids encoding the β-lactamase enzyme by bacteriophages, or by strain dissemination (Berger-Bachi, 2002, Lowy, 2003). Fortunately, in the years following the emergence of penicillin-resistant strains new antibiotics were introduced, including erythromycin, tetracycline and streptomycin which were used to combat *S. aureus*.

1.2.1.2 Streptomycin

Streptomycin, introduced in 1944, was the first aminoglycoside antibiotic to be used in clinical practice. It targets protein synthesis and impairs the proof-reading process by binding to the 30S ribosomal subunit, resulting in the production of defective proteins (Mingeot-Leclercq *et al.,* 1999). Subsequent incorporation of these proteins into the cell wall results in altered permeability and enables increased access of the antibiotic to the cell (Busse *et al.,* 1992). In 1948 resistance to streptomycin was selected *in vitro* by passaging *S. aureus* strains in the presence of increasing drug concentrations and, by 1951, resistance had emerged in clinical isolates of penicillin-resistant *S. aureus* in Australia (Demerec, 1948, Rountree and Barbour, 1951). The mechanism of resistance was proposed to be via an antibiotic modifying nucleotidyltransferase (ANT) enzyme (also known as adenylyltransferase (AAD) enzyme), which impairs the binding of the drug to the ribosome by modifying the streptomycin molecule (Shaw *et al.,* 1993, Suzuki *et al.,* 1975). The *aadE* gene (also known as *ant6* and *ant(6)-Ia*), encoding this adenylyltransferase enzyme, has been associated with streptomycin resistance in staphylococci (Derbise *et al.,* 1996, Shaw *et al.,* 1993). An *aadA* gene (also known as *aad(3")(9)* and *ant(3"-Ia)*) has also been found in *S. aureus* (Courvalin and Fiandt, 1980, Shaw *et al.,* 1993). The *str* gene, thought to encode for an antibiotic modifying phosphotransferase enzyme, is also associated with streptomycin resistance in staphylococci (Ohnuki *et al.,* 1985, Projan *et al.,* 1988). The fact that these resistance genes are associated with plasmids means rapid transfer of resistance occurs and hence the most common form of resistance in *S. aureus* seems to be via enzymatic modification of the drug. However, Lacey *et al.,* (1972) proposed resistance to streptomycin in *S. aureus* could also be due to chromosomal mutations rather than enzymatic inactivation.

1.2.1.3 Chloramphenicol

Chloramphenicol was discovered in 1947 and used soon after for the treatment of infections due to penicillin-resistant *S. aureus*. It is an antibiotic that inhibits bacterial protein synthesis by binding to the peptidyltransferase centre of the 50S subunit of the 70S ribosome (Schwarz *et al.,* 2004). Preliminary work on the resistance mechanism of staphylococci to chloramphenicol

determined that the antibiotic was inactivated in cultures of chloramphenicol-resistant staphylococci (Miyamura, 1964). Suzuki *et al.*, (1966) proposed that chloramphenicol-resistant strains produced an enzyme, which inactivated chloramphenicol through acetylation and furthermore, that acetyl coenzyme A (CoA) was necessary. These findings were later confirmed by Kono *et al.*, (1968) and Shaw *et al.*, (1968). Limited evidence suggested the chloramphenicol acetyltransferase (*cat*) gene was plasmid-mediated (Shaw and Brodsky, 1968). In staphylococci, seven different types of *cat* genes have since been isolated, all of which were present on different plasmids (Schwarz *et al.*, 2004). Ettayebi *et al.*, (1985) demonstrated that resistance to chloramphenicol in a laboratory strain of *E. coli* (*Escherichia coli*) could occur via mutations in the genes encoding 23S rRNA, although this type of resistance mechanism is rarely seen in clinical isolates of *S. aureus*. The toxic effects of chloramphenicol, such as reversible bone marrow suppression and aplastic anaemia, lead to low usage and in a survey carried out in 14 UK hospitals in the early 1970s less than 1% of strains were resistant (Lowbury and Ayliffe, 1974, Yunis, 1989).

1.2.1.4 Tetracycline

The first tetracycline to be discovered was chlortetracycline in 1947, closely followed by oxytetracycline (Roberts, 1996). Tetracycline itself was discovered in 1948 and introduced into clinical use in 1952 (Chopra and Roberts, 2001). It proved to be a useful antibiotic due to its low-cost, broad spectrum and the fact that it can be taken orally (Roberts, 2003, Speer *et al.*, 1992). Tetracycline is a protein synthesis inhibitor. It has been shown to bind strongly to the 30S bacterial ribosomal subunit, preventing the attachment of aminoacyl-tRNA to the ribosome (Epe *et al.*, 1987, Goldman *et al.*, 1983). Although effective for a short while, resistance to tetracycline had emerged by 1953, and by the late 1950s *S. aureus* resistant to both tetracycline and penicillin were common (Watanabe, 1963, Williams, 1959). The mechanism of resistance to tetracycline was determined to be as a result of the acquisition of resistance determinants rather than chromosomal mutations (Roberts, 1996). Resistance is mediated by *tet* (tetracycline) or *otr* (oxytetracycline) genes which encode either efflux pumps, ribosomal protection proteins or modifying enzymes (Roberts, 2003). Thirty-eight *tet* and *otr* genes characterized were recently reviewed (Roberts, 2005). *Staphylococcus* species most commonly carry *tet*(K) and *tet*(L), encoding efflux proteins that

export tetracycline from the cytoplasm, reducing the concentration within the cell. The *tet*(M) and *tet*(O) genes are also commonly found in staphylococci and encode ribosomal protection proteins (Chopra and Roberts, 2001, Roberts, 2005). These bind to the ribosome and block the tetracycline binding site, causing the release of the tetracycline molecule (Roberts, 2005). Efflux genes are usually present on small plasmids, whereas ribosomal protection genes are often present on transposons, which can be integrated into the chromosome, allowing for inter- and intra-species spread by conjugation (Roberts, 2003). The use of tetracycline and oxytetracycline in animal and plant agriculture is thought to have assisted the selection and dissemination of resistance genes (Speer *et al.*, 1992).

1.2.1.5 Macrolides, lincosamides and streptogramins

The macrolides were first introduced in 1952 with the launch of erythromycin. Its lack of cross-resistance with penicillin and the tetracyclines and its activity against gram-positive cocci meant erythromycin was a good antistaphylococcal antibiotic (Thabaut *et al.*, 1985). However, due to the emergence of erythromycin-resistant staphylococci in the United States after extensive use, calls were made for the use of erythromycin in the UK to be restricted to delay the emergence of resistance and preserve the effectiveness of the antibiotic (Anon, 1956, Garrod, 1957, Lepper *et al.*, 1953). Due to this limited-use policy, resistance to erythromycin in staphylococci took slightly longer to emerge than originally anticipated; nevertheless it had occurred in the UK by the late 1950s (Forfar, 1977, Garrod, 1957). Studies on the mechanism of action of erythromycin revealed that it targeted bacterial protein synthesis by binding to the 50S subunit of the ribosome (Vazquez, 1966). Initially, resistance to erythromycin was thought to be via a simple mechanism, however the subsequent introduction of other macrolide antibiotics, spiramycin and oleandomycin, the lincosamide antibiotic, lincosamine, and the streptogramin antibiotics revealed unusual cross-resistance between these groups (Weisblum and Demohn, 1969). All three of these classes of antibiotics, now called the MLS_B (macrolide, lincosamide, $streptogramin_B$) group, share a similar mode of action, spectrum of activity and are affected by a common mechanism of resistance (in addition to mechanisms that affect one class only) (Le Goffic, 1985). This cross-resistance to MLS_B antibiotics is via erythromycin ribosomal methylation (*erm*) genes, which encode an enzyme

that methylates a single residue, A2058, in the 23S rRNA, resulting in reduced binding for MLS antibiotics to the ribosome (Skinner *et al.,* 1983, Weisblum, 1995a). Resistance can be constitutive or inducible. When resistance is inducible, the Erm protein is synthesised only in the presence of a stimulating macrolide, i.e., erythromycin (Fiebelkorn *et al.,* 2003). This results in resistance to 14- and 15-member lactone macrolides, such as erythromycin and azithromycin which are strong inducers, but not to weak inducers, such as 16-member macrolides, such as spiramycin, lincosamides and type B streptogramins (Weisblum, 1995b). When resistance is constitutive, the Erm protein is synthesised constantly, resulting in resistance to all macrolides, lincosamides and type B streptogramins (Weisblum, 1995b). Constitutive resistance is conferred, in staphylococci, by the presence of mutations in the promoter region of the *erm* gene, allowing the production of a methylase without an inducer. Inducible resistance can become constitutive with the emergence of these mutations and with consequent resistance to 16-member macrolides and streptogramin$_B$ antibiotics (Weisblum, 1995b). Both constitutive and inducible strains remain susceptible to type A streptogramins due to their binding at a different site on the ribosome (Vazquez, 1966). *Erm* genes are plasmid mediated, enabling transfer between strains and species (Engel *et al.,* 1980).

Independent mechanisms of resistance to the MLS$_B$ antibiotics have been detected in staphylococci. These include the nucleotidylation gene, *lnu*(A), conferring resistance to lincosamides, the *vat*(A)(B)(C) acetyltransferase genes and the *vgb*(A)(B) hydrolase genes conferring resistance to streptogramins and lincosamides, also the *msr*(A) gene conferring resistance to macrolides and streptogramins via active efflux (Leclercq *et al.,* 1987, Leclercq and Courvalin, 1991, Roberts *et al.,* 1999, Ross *et al.,* 1990, Woodford, 2005).

1.2.2 Emergence of MRSA

This section describes the antibiotic evolution of MRSA and how it has managed to acquire or generate antibiotic resistance mechanisms against nearly all classes of antibiotics. It goes on to discuss the origins of MRSA, their dissemination worldwide, the infections caused by MRSA and the treatment options currently available.

1.2.3 Methicillin resistance in *S. aureus*

In the early 1960s, in an attempt to combat penicillin-resistant strains, semi-synthetic penicillin derivatives such as methicillin, oxacillin, cloxacillin, dicloxacillin and flucloxacillin were manufactured. These antibiotics are not susceptible to the activity of the staphylococcal β-lactamase enzyme (Rolinson, 1998). Nevertheless, the first methicillin-resistant strains were isolated in the UK in 1961, one year after the antibiotic had been introduced (Jevons, 1961). Hartman *et al.*, (1984) discovered the presence of an extra PBP in MRSA, in addition to the three usually found in susceptible *S. aureus*. PBP-2a or -2', as it was named, had a low affinity for methicillin. It was proposed that PBP-2a allowed continued cell wall synthesis in the presence of β-lactam antibiotics (Hartman and Tomasz, 1984). Ubukata *et al.*, (1989) demonstrated that PBP-2a was encoded by the *mecA* gene and suggested accessory genes were involved in its induction. Further work by Ito *et al.*, (1999) and Katayama *et al.*, (2000) determined the *mecA* gene and its accessory genes were located on a staphylococcal cassette chromosome (SCC*mec*). SCC*mec* is a mobile genetic element which is integrated into the chromosome at a specific site, *attBscc* within *orfX* (Hiramatsu *et al.*, 2001). There are five SCC*mec* variants, Types I to V, which vary in size (21-67 Kb) and are classified according to the type of cassette chromosome recombinase gene (*ccr*) present and the organisation of the *mec* complex (Hanssen and Ericson Sollid, 2006).

1.2.4 Origins of MRSA

The origins of MRSA have been much disputed. Lacey *et al.*, (1973) proposed that MRSA evolved from the single acquisition of *mecA*, followed by clonal expansion and diversification. Kreisworth *et al.*, (1993) investigated the presence of the *mecA* gene in 472 MRSA isolates. They concluded that horizontal transfer of *mecA* after its initial acquisition in *S. aureus* is rare (Kreiswirth *et al.*, 1993). Musser *et al.*, (1992), after studying 254 MRSA isolates from nine countries using multilocus enzyme electrophoresis, also concluded that linear descent was probable. Although, subsequent horizontal transfer and recombination events has enabled the distribution of *mecA* to methicillin-susceptible *S. aureus* strains, creating divergent clonal lineages (Musser and Kapur, 1992). More recently, Enright *et al.*, (2002) analysed a collection of 359

MRSA isolates, spanning 38 years and 20 countries, using MLST and SCC*mec* typing to try to determine the origins of MRSA. They found evidence to suggest that successful methicillin-susceptible *S. aureus* (MSSA) clones have acquired the *mecA* gene on more than one occasion, as many isolates had the same sequence type yet different SCC*mec* types. Furthermore, they concluded that MRSA have not evolved from a single clone, but have evolved in relatively few lineages via the horizontal transfer of the *mecA* gene (Enright *et al.,* 2002). Crisostomo *et al.,* (2001) proposed that extensive horizontal transfer, possibly by homologous gene transfer, resulted in the spread of *mecA*. Moreover, it was suggested that the *mecA* gene was acquired before the first reported finding of MRSA in the UK (Crisostomo *et al.,* 2001). The origin of SCC*mec* is not known as it has not been discovered in other genera, however, there is evidence that type I SCC*mec* could have been transferred from *Staphylococcus haemolyticus* (*S. haemolyticus*) (Archer *et al.,* 1996). Additionally, a *mecA* homologue has been identified in *Staphylococcus sciuri* (*S. sciuri*). This does not confer methicillin resistance in *S. sciuri* but does so when transferred into *S. aureus* (Severin *et al.,* 2005, Wu *et al.,* 1996).

1.2.5 Epidemic strains of MRSA

Epidemic strains of MRSA (EMRSA) were defined as those which have been identified in two or more patients in two or more hospitals (Marples *et al.,* 1986). The first UK epidemic MRSA strain, EMRSA-1, was isolated in Essex in 1983, spreading to London in 1984 (Marples *et al.,* 1986). A six month survey by Kerr *et al.,* (1990) confirmed EMRSA-1 had spread to 50 hospitals. A further 13 epidemic strains, EMRSA-2 to -14 were also identified, however, only EMRSA-3 and 12 became widespread in the 1980s and early 1990s (Kerr *et al.,* 1990). Subsequently, two further strains EMRSA-15 and -16 have become dominant. EMRSA-15 emerged in 1991 and is now widespread in most hospitals in the UK. Additionally, it has caused outbreaks in Australia, New Zealand, Germany, Sweden and Finland (Anon, 1992, O'Neill *et al.,* 2001). The emergence of EMRSA-16, in 1992, can be traced back to a single hospital in Northamptonshire (Anon, 1993). By 1994 it had spread to 136 hospitals (Murchan *et al.,* 2004). EMRSA-15 and EMRSA-16 accounted for 96% of MRSA from cases of bacteraemia between 1998 and 2000 (Johnson *et al.,*

2001). There has only been one report of a new epidemic strain since the emergence of EMRSA-15 and -16. Specifically, a study by Aucken *et al.*, (2002) characterized 69 strains isolated between 1997 and 2000 and determined the presence of an epidemic strain, designated EMRSA-17. However, this strain has not proved to be as successful as EMRSA-15 and -16 and remains rare.

1.2.6 Global spread of MRSA

Figures for MRSA bacteraemias are usually presented as a percentage of all *S. aureus* bacteraemias, however, this does not give an indication of the burden of disease. Alternatively, MRSA rates are calculated as the number of MRSA bacteraemias per 1000 bed days. This takes into account the size of the hospital but not whether the hospital has more vulnerable patients nor whether there are infection control measures in place. In the UK, the most recent figures state that 38.9% of *S. aureus* from bacteraemias are due to MRSA (Johnson *et al.*, 2005), whilst the MRSA incidence rate was 0.17 MRSA bacteraemias per 1000 bed days (Johnson *et al.*, 2005). In Europe as a whole, the European Antimicrobial Resistance Surveillance System (EARSS) compared MRSA bacteraemia rates from hospitals across Europe. Results in 2004 and showed MRSA rates were under 10% for The Netherlands, Estonia, Sweden, Finland, Denmark, Iceland and Norway (Figure 2). The highest MRSA rate was seen in Bulgaria at 73%, and 11 European countries had MRSA rates between 25 and 50%. However, the MRSA rates per 1000 bed days for these countries shows a rather different picture (Table 1). Not all of the countries with a high percentage of bacteraemias due to MRSA have a high MRSA rate, e.g., Bulgaria. These data highlight a rough divide between north and south Europe, with a low incidence of MRSA in Scandinavia, when compared with countries like Italy. Further afield, a national survey of 17 Australian hospitals revealed MRSA rates are not as high as in some European countries, with 25% of all bloodstream infections being caused by MRSA (Collignon *et al.*, 2005). A survey of 300 clinical microbiology laboratories in the USA revealed MRSA rates have continued to climb since 1998 with current MRSA bacteraemia rates standing at 49% of all *S. aureus* bacteraemias (Styers *et al.*, 2006). Based on MLST data, Oliveira *et al.*, (2002) identified five major MRSA clones, which accounted for 70% of 3000 isolates from southern and eastern Europe, South America and the USA (Figure 3).

Figure 2. Proportion of invasive *S. aureus* isolates resistant to oxacillin (and equivalents) in 2004 (EARSS data) (Luxembourg (LT), Malta (MT)). (From http://www.rivm.nl/earss/Images/EARSS%20annual%20report%202004%20webversie_tcm61-25345.pdf).

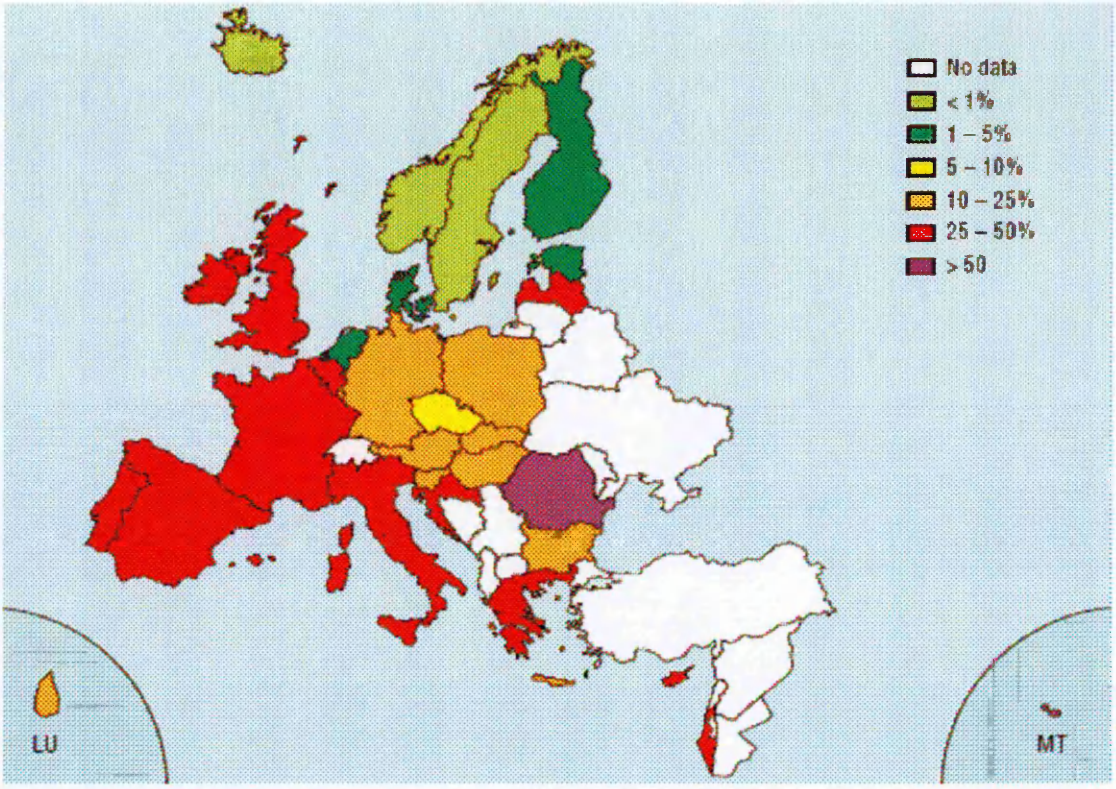


Table 1. Incidence of MRSA bacteraemia per 1000 bed days specified per country in 2004.

(From http://www.rivm.nl/earss/Images/EARSS%20annual%20report%202004%20webversie_tcmtcm61-25345.pdf)

Country	Incidence of MRSA bacteraemia per 1000 bed days
Belgium	0.078
Bulgaria	0.015
Cyprus	0.079
Czech Republic	0.010
Greece	0.033
Estonia	0.003
Spain	0.060
Finland	0.008
France	0.118
Greece	0.074
Croatia	0.061
Hungary	0.013
Ireland	0.120
Israel	0.122
Iceland	0.009
Italy	0.064
Latvia	0.017
Malta	0.193
Netherlands	0.004
Norway	0.001
Poland	0.011
Portugal	0.176
Romania	0.019
Sweden	0.003
Slovenia	0.020
Slovakia	0.020
United Kingdom	0.096

Figure 3. International spread of the pandemic MRSA clones (From Oliveira *et al.*, 2002).

From MLST data they concluded these five pandemic clones, Iberian, Brazilian, Hungarian, New York/Japan and Paediatric, evolved from two genetic backgrounds. EMRSA-15 and -16 do not belong to any of these international clones, however, EMRSA-15 is now spreading in central Europe and may become an international problem. It was proposed that EMRSA-15 and -16 evolved from another two separate lineages (Oliveira *et al.,* 2002).

1.2.7 Infections caused by MRSA

There is no specific 'MRSA disease'. MRSA can infect a range of tissues and organ systems and the range of infections it causes are not matched by any other pathogen. Infection usually begins with colonisation, sometimes followed by local infection, then systemic dissemination usually accompanied by toxinosis (Archer, 1998). Healthy individuals can be colonised by MRSA, usually in the anterior nares or on the skin, without any obvious signs or symptoms of disease, just as with other *S. aureus* strains. Colonisation is thought to precede infection and after the transfer of the bacterium on to the skin, infection can occur through a break in the skin. The infection can remain local causing, for example, cellulitis, wound infection, or can spread to the bloodstream causing bacteraemia or septicaemia (Table 2) (Boyce, 1997). The success of the organism and its ability to cause such a wide range of infections is as a result of its extensive virulence factors which, for example, enable it to thwart phagocytosis, form abscesses, infect tissue from the bloodstream and to invade through tissue from the site of infection (Archer, 1998).

1.2.8 MRSA and nosocomial infection

Infections linked to MRSA as the causative agent have historically been hospital-associated. The transmission of strains between hospitals, cities and countries is usually via colonized or infected patients or by colonised health workers (Boyce *et al.,* 1993). Colonising strains can serve as an endogenous source from which the patient can develop an infection once immunocompromised by surgery or illness. Most transmission is via staff hands although it can be airborne or as a result of direct or indirect contact, for example, environmental surfaces can serve as a reservoir for MRSA strains (Boyce *et al.,* 2005, Shanson and McSwiggan, 1980). Infection control measures are put in

Table 2. Infections for which MRSA is a prominent pathogen (Adapted from Archer, 1998).

Infection	Syndrome
Cellulitis	Inflammation of subcutaneous tissue or muscle
Surgical wound infection	Usually caused by indwelling surgical devices
Pyomyositis	Deep muscle abscesses
Bacteraemia	Presence of bacteria in the bloodstream
Endocarditis	Infection of the inner lining of the heart chambers and/or a heart valve
Osteomyelitis	Inflammation of bone as a result of infection
Empyema	Accumulation of pus in a body cavity, usually thoracic cavity
Septicaemia	Dissemination of infection throughout the body, causing septic shock
Scalded skin syndrome	A side effect of infection, sloughing of the skin, blistering
Toxic shock syndrome	Blood-borne bacterial infection

place to reduce the risk of transmission, including the eradication of nasal carriage pre-hospital admission or pre-surgery, i.e., use of mupirocin, use of alcohol hand rubs, isolation of contaminated patients and screening of staff and patients. However, these control measures rely on the availability of isolation rooms and the compliance of hospital staff, patients and visitors (Coia *et al.*, 2006). The use of mupirocin to eradicate nasal colonisation has resulted in increased resistance to this antibiotic (Marples *et al.*, 1995). Epidemic strains, such as EMRSA-15 and -16, are still commonplace in many of Britain's hospitals, despite the introduction of national control measures (Coia *et al.*, 2006).

1.2.9 Multi-resistant MRSA

Many strains of MRSA are resistant to other antibiotics, not just methicillin. The next section focuses on the antibiotics these strains are resistant to and the mechanisms involved. Most EMRSA-15 and -16 strains are only resistant to β-lactam, fluoroquinolones and macrolide antibiotics, however some MRSA strains are resistant to other classes of antibiotics as well.

1.2.9.1 Gentamicin

Gentamicin, introduced in the 1958, was the fourth antibiotic to be introduced from the aminoglycoside family; streptomycin, neomycin and kanamycin were already in use. Treatment of MRSA infections with gentamicin was initially successful and may have been the cause of a decline in MRSA rates in the mid-1970s (Johnson *et al.*, 2005). Nevertheless, resistance to gentamicin had been observed as early as 1969 and, by the mid-1980s, resistance was widespread (Graham, 1981, King *et al.*, 1981, Lacey and Mitchell, 1969). Gentamicin differs in its molecular structure from streptomycin and, although they have a similar mode of action and resistance is mediated by modifying enzymes in both cases, there is no cross-resistance between the two antibiotics (Phillips and Shannon, 1984, Shaw *et al.*, 1993). Gentamicin resistance is primarily mediated by inactivating enzymes that modify amino and hydroxyl groups at different sites of the antibiotic. There are three classes of these aminoglycoside-modifying enzymes; *N*-acetyltransferases (ACC) which modify amino residues, *O*-nucleotidyltransferases (ANT) and *O*-

phosphotransferases (APH) which modify hydroxyl sites (Mingeot-Leclercq *et al.,* 1999, Phillips and Shannon, 1984). Antibiotic modification results in a drug that binds poorly to the ribosome. The genes encoding the drug modification enzymes are plasmid-encoded, but are associated with transposable elements, facilitating rapid transfer (Mingeot-Leclercq *et al.,* 1999). Jaffe *et al.,* (1980) showed interspecies transfer of gentamicin-resistance plasmids between *S. aureus* and *S. epidermidis*.

Gentamicin resistance rates in MRSA strains have declined, possibly due to clone replacement. A British Society for Antimicrobial Chemotherapy (BSAC) survey in 2004 revealed only 5% of MRSA strains isolated from bacteraemias were resistant to gentamicin (http:// www.bsacsurv.org/ mrsweb/bacteraemia).

1.2.9.2 Fluoroquinolones

A new antibiotic class was discovered in the early 1960s with the introduction of the first synthetic quinolone, naladixic acid (Wolfson and Hooper, 1989). Quinolones were made more potent by fluorination of the 6-position, producing the fluoroquinolones. The first fluoroquinolone, flumequine, was short-lived due to toxicity problems, however a second-generation fluoroquinolone, ciprofloxacin, was developed via modification of side chains in the early 1980s (Ball, 2000). Other second-generation fluoroquinolones, norfloxacin and ofloxacin, were developed soon after. Ciprofloxacin showed good activity against MRSA and was launched in the mid-1980s (Smith and Eng, 1985). However, resistance to ciprofloxacin in staphylococci occurred almost immediately and calls were made for discretion when using the antibiotic to avoid the rapid development of resistance (Smith *et al.,* 1985). New, more potent, fluoroquinolones have been developed including levofloxacin, sparfloxacin, moxifloxacin, gatifloxacin, gemifloxacin and more recently, sitafloxacin, garenoxacin and fandofloxacin (Ball, 2000, Woodford, 2005).

Fluoroquinolones target two essential enzymes; DNA gyrase and DNA topoisomerase IV; the latter is the main target in *S. aureus* (Ferrero *et al.,* 1994). Mutations in the genes encoding these enzymes result in amino acid substitutions and consequently modification of the target site. Mutations in the genes encoding DNA gyrase, *gyr*A and *gyr*B, and the genes encoding subunits of DNA topoisomerase IV, *grl*A and *grl*B, have been detected in staphylococci (Ferrero *et al.,* 1995,

Fournier and Hooper, 1998, Ito *et al.,* 1994). These mutations can be present singularly or in combination, with certain double combinations conferring high-level resistance (Wang *et al.,* 1998). In the UK, in 2004, a BSAC survey showed all MRSA isolates, from bacteraemias, were resistant to ciprofloxacin (http://www.bsacsurv.org/ mrsweb/bacteraemia).

1.2.9.3 Rifampicin

The discovery of the rifamycin class of antibiotics in 1957 led to the development of a semi-synthetic derivative called rifampicin (also known as rifampin, rimactane and rifadin) (Wehrli and Staehelin, 1971). It showed particular promise against strains of *S. aureus* and, additionally, its novel mechanism of action meant there is no cross-resistance with any other class of antibiotic (Dixson *et al.,* 1984, Kunin *et al.,* 1969). Rifampicin works by binding to RNA polymerase, specifically to the β subunit, preventing the initiation of messenger RNA (mRNA) synthesis (Morrow and Harmon, 1979). In staphylococci, resistance to rifampicin is via mutations in the *rpoB* gene, which encodes the RNA polymerase β subunit. This results in the antibiotic binding to the mutated enzyme with less affinity (Morrow and Harmon, 1979). Levels of resistance depend upon both the location and the nature of the amino acid substitution (Aubry-Damon *et al.,* 1998). In-vitro studies warned of the ease with which rifampicin-resistant mutants of *S. aureus* arose (at a frequency of 10^{-7}-10^{-8}) and advised using this antibiotic in combination with another agent (Kunin *et al.,* 1969, Morrow and Harmon, 1979). This finding was echoed in the clinic where resistance occurred rapidly when large numbers of bacteria (>10^{6}) were present (Kapusnik *et al.,* 1984). Rifampicin was not widely used for the treatment of staphylococcal infections when it was first introduced due to the availability of other, more widely used antibiotics (Dixson *et al.,* 1984). However, despite its use in combination with other antibiotics resistance still emerged in the clinic (Simon *et al.,* 1983). Today the use of rifampicin is still advocated, however, only in combination with glycopeptide antibiotics, fusidic acid, fluoroquinolones or trimethoprim (Gemmell *et al.,* 2006).

1.2.9.4 Fusidic Acid

Fusidic acid is an antibiotic that inhibits bacterial protein synthesis. It binds to the elongation factor G and prevents the binding of transfer RNA (tRNA) to the ribosome, thereby preventing elongation of the peptide chain (Tanaka *et al.,* 1968). Fusidic acid was launched in 1962 for use as a topical agent as well as a systemic antibiotic. It showed particularly good activity against staphylococci and only had cross-resistance with one other antibiotic, cephalosporin P_1 (an antibiotic structurally related to fusidic acid, not traditional cephalosporins) (Godtfredsen *et al.,* 1962). *S. aureus* mutants resistant to fusidic acid were easily selected *in vitro* (at frequencies of 10^{-6}-10^{-8}) (Barber and Waterworth, 1962, Evans and Waterworth, 1966, Hilson, 1962, Lacey and Grinsted, 1972). Resistant *S. aureus* strains were encountered in the clinic almost immediately (Lowbury *et al.,* 1962). Resistance has been attributed to mutations in the *fusA* gene, which encodes for the elongation factor G. Different mutations are thought to confer different levels of resistance and multiple mutations within the same region can occur (Besier *et al.,* 2003). Lacey *et al.*, (1974) proposed fusidic acid resistance could also occur via a plasmid-mediated mechanism. Recent investigations have confirmed that a plasmid-mediated gene, *fusB*, confers resistance in clinical isolates of *S. aureus*, however the mode of action of this gene is still to be elucidated. O'Neill *et al.*, (2006) suggested that the binding of FusB to the target site protects the translational apparatus from inhibition by fusidic acid. Lacey *et al.*, (1972) suggested plasmid-mediated resistance was a more predominant mechanism of resistance than chromosomal mutation in clinical isolates, a finding confirmed by O'Neill *et al.*, (2004). It has been proposed that these two mechanisms of resistance are incompatible as they have not yet been discovered within the same isolate (O'Neill and Chopra, 2006). Additionally, other mechanisms of resistance may also be possible as proved by the isolation of clinical specimens with neither *fusA* mutations or a *fusB* gene (O'Neill *et al.,* 2004). Resistance to fusidic acid, in MRSA bacteraemias, has remained relatively low, given the rapid emergence of resistant mutants *in vitro* and in the clinic. This could be attributed to the limited use of fusidic acid, given the ease with which resistance occurs, or the fact that it is usually used in combination therapy with other antibiotics to delay the emergence of resistance (Besier *et al.,* 2003). Current guidelines for the treatment of MRSA advise fusidic acid only to be used in combination with other antibiotics (Gemmell *et al.,* 2006). Resistance rates

show 8% of MRSA strains isolated from bacteraemias to be resistant to fusidic acid (http://www.bsacsurv.org/mrsweb /bacteraemia). However, although resistance is infrequent in bacteraemia isolates, widespread use of fusidic acid as a topical agent to combat clones causing dermatological conditions, such as impetigo bullosa, has resulted in increased resistance rates (Brown and Thomas, 2002, O'Neill *et al.*, 2004).

1.2.9.5 Vancomycin

Vancomycin, a glycopeptide antibiotic, is one of the mainstays of current anti-MRSA therapy. Discovered in 1951, unlike other antibiotics launched at the same time, it still remains almost universally effective against MRSA. Vancomycin exerts its action by preventing the attachment of precursors into a nascent peptidoglycan chain, thus preventing successful cell wall synthesis (Reynolds, 1989).

The first clinical cases of vancomycin-resistant enterococci (VRE) were described in 1988 (Uttley *et al.*, 1988). In enterococci, resistance is associated with the presence of operons that encode a series of Van enzymes which, firstly, are responsible for the synthesis of low-affinity precursors resulting in a modified target, and secondly, eliminate the un-modified precursors, which have a high affinity for vancomycin (Arthur *et al.*, 1996). Six Van variants have been identified in enterococci one of which, *vanA*, is associated with providing high level inducible resistance to both vancomycin and teicoplanin, however not all *van* systems confer resistance to both vancomycin and teicoplanin. Five of the six Van variants (*A, B, D, E* and *G*) are plasmid-mediated, whereas *vanC* is intrinsic to certain enterococcal species (Courvalin, 2006).

The transfer of Van genes from enterococci to staphylococci has long been feared. This was proved possible *in vitro* by Noble *et al.*, (1992) who managed to transfer high-level resistance from a strain of *Enterococcus faecalis* (*E. faecalis*) to *S. aureus*. It was also implied that transfer was transposon-mediated (Noble *et al.*, 1992). However, this became a reality in the clinic with the emergence of vancomycin-resistant strains. Within a four month period in 2002, two unrelated isolates, fully vancomycin-resistant MRSA (minimum inhibitory concentrations (MICs) 1028 mg/L and 32 mg/L) were isolated from patients in hospitals in Michigan and Pennsylvania, USA (Anon, 2002a, Anon, 2002b, Clark *et al.*, 2005). A third vancomycin-resistant *S. aureus* (VRSA) strain

occurred in 2004 in a hospital in New York and a fourth was isolated in Michigan in 2005 (Anon, 2004, Appelbaum and Jacobs, 2005). Two more have since been isolated in Michigan, bringing the total to six (http://www.cdc.gov/ ncidod/dhqp/ar_visavrsa_FAQ.html).

Investigation of the clinical isolates from Michigan and Pennsylvania revealed the presence of *vanA* genes identical to that found in VRE (Clark *et al.,* 2005, Tenover *et al.,* 2004, Weigel *et al.,* 2003). The Michigan strain harboured a plasmid that contained genetic sequence identical to Tn*1546*, the transposon containing the *vanA* gene found in enterococci (Weigel *et al.,* 2003). The Pennsylvania strain harboured a large plasmid with some sequence homology to Tn*1546*, although not to the same extent as the first Michigan strain. The size of this plasmid and the level of expression of the *vanA* gene could be a reason for the relatively low MIC encountered in this strain when compared with that of the Michigan strain (32 mg/L cf. 1024 mg/L) (Tenover *et al.,* 2004). In the Michigan isolate, vancomycin resistance seemed to be due to *in vivo* transfer from enterococci to staphylococci in a mixed infection (Weigel *et al.,* 2003). It was proposed that the Van genes had transferred to a resident staphylococcal plasmid (Weigel *et al.,* 2003). The degree with which these two plasmids differed both from each other and from Tn*1546* indicates that the acquisition of the *vanA* genes occurred independently in the two strains (Clark *et al.,* 2005). These strains were epidemiologically unlinked, although both were USA 100 (via PFGE typing), one of the predominant MRSA types in the USA (also known as the New York/Japan clone) (Tenover *et al.,* 2004, Weigel *et al.,* 2003). Following the isolation of these two strains, the main cause for concern is the dissemination of vancomycin resistance among clones of the predominant epidemic MRSA strains. Worryingly, the Pennsylvania strain was isolated from a patient not on vancomycin therapy, indicating resistance can be selected for in the absence of vancomycin (Whitener *et al.,* 2004).

Vancomycin intermediate-resistant *S. aureus* (VISA) strains are associated with heteroresistance in the clinic, although homoresistance does also occur (Hiramatsu *et al.,* 1997, Kim *et al.,* 2002, Sng *et al.,* 2005). Heteroresistant strains appear susceptible, but contain subpopulations that are able to grow at vancomycin concentrations of 4 mg/L or greater (Hiramatsu *et al.,* 1997). In both hetero- and fully-resistant strains resistance has been attributed to significantly increased cell wall synthesis, which serves as a physical barrier against the penetration of vancomycin molecules (Cui

et al., 2000). It has been proposed that cell wall thickening is associated with the increased production of high-affinity peptidoglycan subunits, which capture and immobilise vancomycin molecules before they reach the sites of cell-wall synthesis (Sieradzki and Tomasz, 1999). However, the exact mechanism that leads to this cell wall thickening has still to be elucidated. VISAs are rare and there are few reports of VISAs occurring in the UK (Howe *et al.,* 1998). None were isolated in the UK 2004 BSAC bacteraemia survey (http://www.bsacsurv.org/mrsweb /bacteraemia).

1.2.9.6 Teicoplanin

Teicoplanin was the second glycopeptide antibiotic to be introduced. It is structurally related to vancomycin and has similar properties, although its mode of action differs slightly. Like vancomycin, teicoplanin binds to the cell wall precursors and inhibits their incorporation into a nascent peptidoglycan chain, thus preventing successful cell wall synthesis (Greenwood, 1988). Unlike vancomycin, teicoplanin has a hydrophobic tail which anchors it to the cell membrane, close to its target (Van Bambeke, 2004). Resistance to teicoplanin was first recognised in 1986, six years after the discovery of the compound, in a clinical isolate of *S. haemolyticus* (Wilson *et al.,* 1986). Resistance to teicoplanin in *S. aureus* emerged in 1990 (Kaatz *et al.,* 1990). Resistance to teicoplanin in staphylococci is thought to be via a shift in cell wall composition, providing cell wall precursors with an increased capacity for binding teicoplanin molecules. These sequestered antibiotic molecules may sterically hinder the access of incoming molecules (Sieradzki and Tomasz, 1998). In addition Sieradzki *et al.,* (1998) and Bischoff *et al.,* (2001, 2001) have associated teicoplanin resistance with the *sigmaB* factor, which is involved in transcription, though its exact role in resistance is still unclear. It has been proposed that multiple alterations in the cell wall structure and synthetic machinery, acquired through sequential mutational steps, may be required (Bischoff *et al.,* 2001, Sieradzki and Tomasz, 1998). Resistance to teicoplanin provides no cross-resistance to any other antibiotics, however, the VanA phenotype does provide resistance to both vancomycin and teicoplanin (Courvalin, 2006, Wilson *et al.,* 1986). Currently, resistance to teicoplanin in clinical isolates of *S. aureus* in the UK is rare, for example, no isolates with intermediate or full resistance were found in the 2004 BSAC bacteraemia survey, although many

isolates of EMRSA-17 are teicoplanin-resistant (Manuel *et al.,* 2002). Resistance is more commonly associated with coagulase-negative staphylococci. BSAC data from 2004 revealed 5% of *S. epidermidis* and 30% of *S. haemolyticus* bacteraemia isolates from the UK showed intermediate resistance to teicoplanin (http://www.bsacsurv.org/mrsweb/bacteraemia). Why CNS resistance rates are higher than *S. aureus* is unknown, it is possibly due to intrinsic resistance (Lallemand *et al.,* 2002).

1.2.9.7 Mupirocin

Mupirocin, previously known as pseudomonic acid, was first isolated in 1971 but was not introduced into the UK until 1985 (Cookson *et al.,* 1990, Fuller *et al.,* 1971). It has an unique mechanism of action, is highly active against staphylococci and is used as a topical agent (Casewell and Hill, 1985, Sutherland *et al.,* 1985). Mupirocin binds to the isoleucyl tRNA synthetase and prevents the incorporation of isoleucine into a nascent peptide chain, thereby halting protein synthesis (Casewell and Hill, 1987). Additionally, the absence of protein synthesis, and in particular of tRNA charging, results in the initiation of the stringent response, (a mechanism by which bacteria can regulate transcription during amino acid starvation) and the shutdown of RNA synthesis (Cassels *et al.,* 1995, Hughes and Mellows, 1978). Mupirocin is used for the topical treatment of bacterial skin infections and for the eradication of nasal carriage of MRSA (Dacre *et al.,* 1983, Sutherland *et al.,* 1985). Although resistance was encountered in laboratory studies, mupirocin-resistant staphylococci did not emerge in the clinic until 1987 (Rahman *et al.,* 1987, Sutherland *et al.,* 1985). Low-level mupirocin resistance (MICs 8-256 mg/L) is associated with chromosomal mutations in the *ileS* gene which encodes for isoleucyl tRNA synthetase (Antonio *et al.,* 2002). High-level resistance (MICs $\geq$ 512 mg/L) is via the *mupA* gene, which encodes an alternative mupirocin-resistant isoleucyl tRNA synthetase, which replaces the native isoleucyl tRNA synthetase (Gilbart *et al.,* 1993, Woodford *et al.,* 1998). The *mupA* gene, previously referred to as the *ileS* or *ileS2* gene, is plasmid-mediated, enabling inter-strain and -species spread (Antonio *et al.,* 2002, Bradley *et al.,* 1995, Hodgson *et al.,* 1994). It has only 52% identity with the native *ileS* gene of *S. aureus*, indicating that it has not arisen by mutations to the native gene; rather it has probably been acquired from another organism (Hodgson *et al.,* 1994). Evidence for the

transmission of the *mupA* gene between species is provided by Hurdle *et al.*, (2005) and Leski *et al.*, (1999), who showed that transfer between *S. epidermidis* and *S. aureus* in the clinic was possible.

1.2.9.8 Fosfomycin

Fosfomycin is a broad-spectrum antimicrobial, derived from several species of *Streptomyces* (*S. fradiae*, *S. viridochromogenes* and *S. wedmorensis*), which inhibits cell wall synthesis at an early stage (Stapley *et al.*, 1969). Fosfomycin is taken into the cytoplasm by the L-α-glycerolphosphate pathway or the hexose phosphate uptake (Uhp) system, which are active transport systems induced by glycerol-6-phosphate (G-6-P) (Kahan *et al.*, 1974). Resistance to fosfomycin is determined by the plasmid-mediated *fosB* gene in gram-positive organisms (Etienne *et al.*, 1989, Etienne *et al.*, 1991). The exact mechanism of resistance conferred by *fosB* is unclear, but is thought to be similar to *fosA*, the resistance gene in gram-negative species, which modifies the antibiotic by opening the epoxide group (Arca *et al.*, 1988). Additionally, chromosomal mutations in *glpT* or its regulatory genes result in modifications to the L-α-glycerolphosphate pathway and resistance to fosfomycin (Nilsson *et al.*, 2003). Resistant mutants can be made more susceptible *in vitro* in the presence of G-6-P which induces the secondary transport system, the Uhp pathway, and hence the uptake of fosfomycin (Kahan *et al.*, 1974). Similarly, mutations in the *uhpT* gene and its regulatory proteins also confer resistance to fosfomycin. Fosfomycin is licensed for clinical use in the UK but is not marketed. It has been used more widely abroad (Etienne *et al.*, 1991).

1.2.10 New agents for the treatment of MRSA

Linezolid (see section 1.3), vancomycin and teicoplanin are currently the antibiotics of choice for the treatment of MRSA bacteraemias, pneumonias, serious soft tissue infections, bone and joint infections and, occasionally, in surgical site prophylaxis either singularly or in combination with other antibiotics (Gemmell *et al.*, 2006). However, due to the steady emergence of antibiotic-resistant pathogens, the development of new antimicrobials to target MRSA essential.

Dalbavancin, oritavancin and telavancin are all new glycopeptide antibiotics (Table 3). Dalbavancin is a semi-synthetic teicoplanin derivative. It shows activity against MRSA, VISA, glycopeptide-susceptible strains and glycopeptide-resistant enterococci (GRE) strains, however is not effective against GRE strains with VanA (Candiani *et al.,* 1999, Lin *et al.,* 2005). As a result it probably would not be effective against VRSA strains with a *vanA* genotype. Dalbavancin is most notable for having a serum half-life of over one week, enabling flexible and, possibly, once weekly dosing regimens (Woodford, 2005). Oritavancin is a semi-synthetic glycopeptide with activity against MRSA and GRE strains (Garcia-Garrote *et al.,* 1998, Schwalbe *et al.,* 1996). However, questions concerning its longevity in the clinic have been made after oritavancin-resistant enterococci were selected *in vitro* (Arthur *et al.,* 1999). Telavancin is a lipoglycopeptide antibiotic that is proposed to have two mechanisms of action. It inhibits cell wall synthesis and also interacts with the bacterial membrane, dissipating the membrane potential and causing changes in cell permeability (Higgins *et al.,* 2005). Resistance should be slower to emerge as multiple mechanisms of action may mean multiple resistance mechanisms are necessary (Krause *et al.,* 2003). Both telavancin and oritavancin show activity against strains of MRSA, glycopeptide-intermediate *S. aureus*, VRSA and VRE (Higgins *et al.,* 2005, King *et al.,* 2004). Telavancin is currently in phase III clinical trials.

Tigecycline is a glycylcycline, structurally related to the tetracyclines. It is effective against highly-resistant gram-positive bacteria, including MRSA, and is not subject to the same resistance mechanisms affecting existing tetracyclines (Livermore, 2005). However, serial passage of *S. aureus* in the presence of increasing concentrations of tigecycline resulted in resistant mutants due to the over expression of a efflux pump, *mepA* (McAleese *et al.,* 2005). Additionally, reports of variant *tet* genes, able to confer resistance to glycylcyclines, being isolated in veterinary isolates of *Salmonella* (Tuckman *et al.,* 1998) and the discovery of resistance to glycylcyclines in *Salmonella* and *Shigella* isolates from Africa is of concern (Chopra and Roberts, 2001). Daptomycin (Cubicin) is the first member of a new class of bactericidal antibiotics called the lipopeptides and is effective against nearly all clinically relevant gram-positive bacteria (Steenbergen *et al.,* 2005). Daptomycin inserts itself into the cell membrane, causing depolarisation. This itself is not lethal, but the secondary effects are. This mechanism of action is unique from all other drugs currently available,

Table 3. A comparison of new anti-MRSA antibiotics.

Antibiotic	Antibiotic class	No. of staphylococci tested[A]	MIC_{50} (mg/L)	MIC_{90} (mg/L)	Reference
Ceftobiprole	Cephalosporin	303	0.125 - 2	0.5 - 2	(Bogdanovich *et al.*, 2005)
Dalbavancin	Lipoglycopeptide	146	0.03 - 0.06	0.06	(Lin *et al.*, 2005)
Daptomycin	Cyclic lipopeptide	146	0.25 - 0.5	0.5	(Lin *et al.*, 2005)
Oritavancin	Glycopeptide	146	2	2 - 4	(Lin *et al.*, 2005)
Telavancin	Lipoglycopeptide	145	0.25 - 0.5	0.25 - 0.5	(King *et al.*, 2004)
Tigecycline	Glycylcycline	892	0.25 - 0.5	0.25 - 1	(Reynolds *et al.*, 2004)
Iclaprim	Dihydrofolate reductase (DHFR)	100	0.12 - 0.25	0.5 - >32	(Bajaksouzian *et al.*, 2002)
Ceftaroline	Cephalosporin	N/A	N/A	N/A	N/A

[A] Includes MRSA, MSSA and coagulase-negative species.

and consequently daptomycin may be able to circumvent existing resistance mechanisms (Silverman *et al.,* 2003). Resistance to daptomycin in *S. aureus* nevertheless can be selected by serial passage *in vitro*, although the mechanism of resistance is unknown (Silverman *et al.,* 2001). Furthermore, daptomycin is a natural product, isolated from *S. roseosporus*, and therefore there is the possibility that resistance genes could exist in this species and transfer to clinically-significant species (D'Costa *et al.,* 2006, Woodford, 2003).

Ceftobiprole is a new "fifth-generation" cephalosporin. Unlike other cephalosporins, so far marketed, it shows good activity against MRSA due to its increased affinity for PBP2a, which it binds and inactivates (Bogdanovich *et al.,* 2005, Hebeisen *et al.,* 2001, Jones *et al.,* 2002). It is possible to generate low-level resistance in staphylococci *in vitro* via serial passage in the presence of ceftobiprole, however the mechanism by which this occurs is unknown (Bogdanovich *et al.,* 2005). Another new cephalosporin which is active against strains of MRSA is ceftaroline (PPI-0903) (Andes and Craig, 2006). It has recently entered phase III trials and the USA Food and Drug Administration (FDA) granted it fast-track designation for the treatment of cSSSI (complicated skin and skin structure infections) caused by MRSA (http://www.cerexa.com/press/040506pr.html).

A new antibiotic currently in the pipeline is iclaprim. Iclaprim is a novel, synthetic inhibitor of DHFR which shows activity against MRSA (Bajaksouzian *et al.,* 2002, Then *et al.,* 2002). Nevertheless, its usefulness against MRSA strains is potentially impaired by the fact that there is a degree of cross-resistance between trimethoprim (another DHFR inhibitor) and iclaprim (Then *et al.,* 2002). Iclaprim is currently undergoing phase III trials for the treatment of cSSSI also after being granted fast track status by the USA FDA (http://iclaprim.com/).

Several new oxazolidinones were investigated after the launch of linezolid in the hope of increasing their activity and spectrum. Those currently in development include VIC-105535 (Pfizer), RWJ-416457 (Johnson & Johnson), Rx-01 (Rib-X Pharmaceuticals) and DA-7157 (Dong-A Pharm). These new compounds show activity comparable to or slightly greater than that of linezolid (Choi *et al.,* 2005, Foleno *et al.,* 2005, Lawrence *et al.,* 2005, Luehr *et al.,* 2005).

1.2.11 Community-acquired MRSA

The deaths of four healthy children in Dakota and Minnesota (USA) in 1999 reflected the emergence of community-acquired MRSA (CA-MRSA) capable of causing severe infections (Anon, 1999). Early CA-MRSA strains in the USA were recognised as being only resistant to methicillin and other β-lactam antibiotics due to the presence of the SCC*mec* type IV element. This element is smaller and may be more mobile than its counterparts, SCC*mec* types I-III. It does not carry resistant determinants to non-β-lactam antibiotics (Daum *et al.,* 2002). Multiply resistant, highly-virulent and invasive CA-MRSA epidemic clones, such as the USA 300 strain, have since emerged (Diep *et al.,* 2006, Vandenesch *et al.,* 2003). Lina *et al.*, (1999a) made the connection between CA-MRSA and the Panton-Valentine leukocidin (PVL) virulence factor, detecting PVL genes in 23 of 27 CA-MRSA isolates. CA-MRSA strains with PVL genes were subsequently associated with necrotic lesions of the skin and subcutaneous tissues and with severe community-acquired necrotic pneumonia (Lina *et al.,* 1999a). Vandenesch *et al.*, (2003) investigated 117 CA-MRSA isolates in three continents and were able to elucidate that both SCC*mec* type IV element and the PVL locus were prevalent in (but not exclusive to) CA-MRSA isolates. Additionally, they proposed that the emergence of CA-MRSA strains did not reflect the dissemination of one clone but was due to the independent worldwide evolution of multiple CA-MRSA strains, not related to local hospital MRSA strains. The significance and genetic advantage conferred by the presence of the PVL genes and the SCC*mec* type IV element has not yet been proved or disproved (Vandenesch *et al.,* 2003). Although these CA-MRSA strains seem to be well suited to the community environment, the detection of CA-MRSA strains with PVL genes and that are related to the two major clones in UK hospitals, EMRSA-15 and EMRSA-16, is a major cause for concern and presents a real and potential threat (Holmes *et al.,* 2005).

1.3 Oxazolidinones

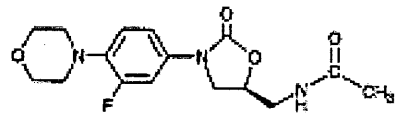
1.3.1 Background

Researchers at E.I. du Pont de Nemours & Co. originally developed oxazolidinones as monoamine oxidase inhibitors for the treatment of depression. It was subsequently discovered that they had antimicrobial activity and they were developed for use in the control of bacterial plant diseases in the late 1970s (Brickner, 1996). In 1987, improvement of these initial compounds resulted in the development of the first two oxazolidinone analogues for use against human pathogens, DuP 721 and DuP 105 (Slee *et al.,* 1987). These analogues showed promise *in vitro* but lethal toxicity in animals prevented their development (Brickner, 1996, Slee *et al.,* 1987).

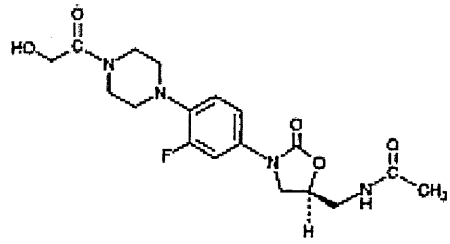
Preliminary research showed oxazolidinones to be active against problem gram-positive bacteria, including MRSA and enterococci and mutant generation was difficult. They were water-soluble and had a novel mechanism of action, unlike existing antibiotic classes (Brumfitt and Hamilton-Miller, 1988, Daly *et al.,* 1988, Slee *et al.,* 1987). These promising attributes ensured work continued on these compounds by researchers at Upjohn Laboratories. In 1996 their perseverance was rewarded with the development of two non-toxic derivatives, linezolid (UP-100176) and eperezolid (UP-100592) (Figure 4) (Brickner *et al.,* 1996). Linezolid was preferred for clinical use due to its better pharmacokinetic and safety profile (Barrett, 2000). Following FDA approval, linezolid was launched in the USA in May 2000 (Hamel *et al.,* 2000). Subsequently, it was launched in the UK in January 2001 and is currently being marketed under the trade name Zyvox. It is licensed for the treatment of VRE infections, nosocomial infections caused by *S. aureus* or penicillin-susceptible *Streptococcus pneumoniae* (*S. pneumoniae*), cSSSIs caused by *S. aureus*, *Streptococcus pyogenes* or *Streptococcus agalactiae* and community-acquired pneumonia due to *S. aureus* (MSSA only) or penicillin-susceptible *S. pneumoniae* (Barrett, 2000).

Figure 4. Chemical structures of linezolid and eperezolid. **(A)** Linezolid **(B)** Eperezolid (From Shinabarger *et al.,* 1997).

(A)



(B)

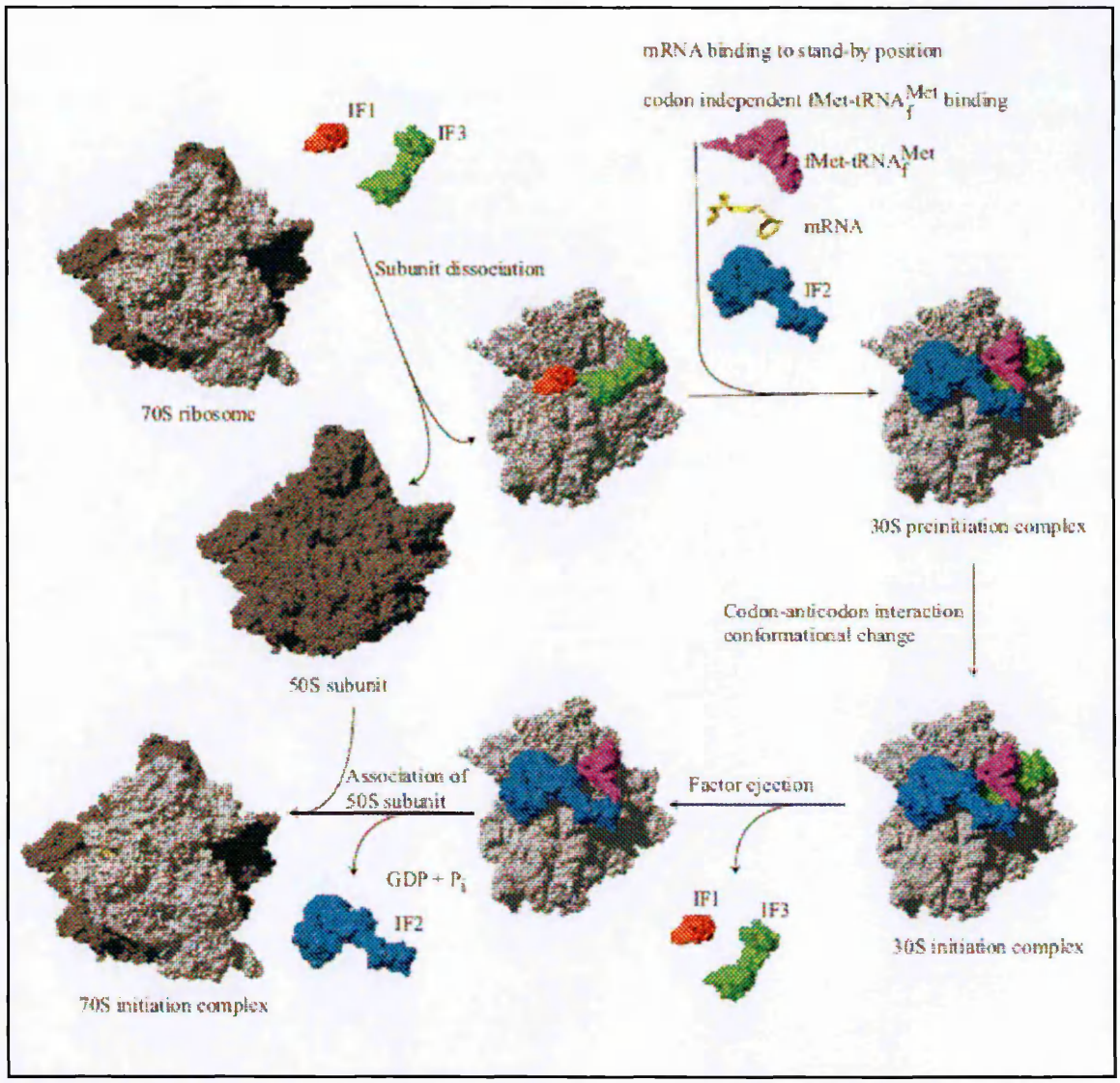


1.3.2 Mechanism of action of linezolid

Bacterial protein synthesis involves, firstly, the assembly of a functional ribosome on an mRNA ready to commence protein synthesis (initiation). Next the correct amino acid is brought to the ribosome (via tRNA), joined to the nascent polypeptide chain and the entire assembly translocates along the mRNA (elongation). When a stop codon is reached the newly-synthesized polypeptide is released from the ribosome (termination). The ribosome releases mRNA and tRNA and is recycled.

The initiation stage of protein synthesis requires the assembly of several components (the 70S ribosome, three initiation factors (IF1, IF2, IF3) and initiator tRNA (N-formyl-methionyl-tRNA ($tRNA_f^{Met}$)) into a functionally competent ribosome on an mRNA strand ready to commence protein synthesis (Figure 5). The 70S subunit is comprised of 50S and 30S subunits and has three tRNA binding sites, aminoacyl (A), peptidyl (P) and exit (E) (Laursen *et al.*, 2005). Binding of IF3 to the ribosome promotes dissociation into its two component subunits, the 30S (comprised of 16S rRNA and 21 proteins) and 50S (comprised of 5S, 23S rRNA and 34 proteins) subunits (Petrelli *et al.*, 2001). IF1 guides the initiator tRNA to its correct binding site, the peptidyltransferase P site, on the 30S subunit, forming a complex comprising of the 30S subunit, three initiation factors, mRNA and $tRNA_f^{Met}$. Initiation factors IF1 and IF3 dissociate and IF2 promotes the incorporation of the 50S subunit into the complex. As the 50S subunit of the ribosome associates with the 30Sinitiation complex, hydrolysis of GTP bound to IF2 occurs. Subsequent dissociation of IF2 leaves a 70S initiation complex and translation can proceed (Laursen *et al.*, 2005). Initial studies on oxazolidinones concluded that their target site was protein synthesis, however, conflicting research emerged as to the exact mechanism of action (Slee *et al.*, 1987). Eustice *et al.*, (1988) determined that oxazolidinones must inhibit an early event in initiation, preceeding the interaction between $tRNA_f^{Met}$ and the 30S subunit (Eustice *et al.*, 1988). Later research by Lin *et al.*, (1997) found that eperezolid bound to the 50S subunit near to the interface with the 30S subunit, possibly preventing the formation of a functional 70S initiation complex whilst Swaney *et al.*, (1998a) determined that oxazolidinones inhibited development of the initiation complex by preventing formation of the $tRNA_f^{Met}$-70S and 30S subunit-mRNA complex.

Figure 5. Translation initiation pathway in bacteria. The 30S and 50S subunits are shown in light and dark grey respectively. Translation factors IF1, IF2 and IF3, mRNA and fMet-tRNA$_f^{Met}$ are shown in red, blue, green, yellow and purple, respectively (Diagram from Laursen *et al.*, 2005).



Continued research led to the finding that oxazolidinones bound to domain V, the 2100-2180 region, of the 50S ribosomal subunit. Other antibiotics targeting the ribosome have not been associated with binding to this region, indicating why the mechanism of action of oxazolidinones was unique (Matassova *et al.,* 1999). Confirmation of the interaction of oxazolidinones with the 50S subunit was provided by the discovery of mutations in the peptidyl transferase domain of the genes encoding 23S rRNA in a laboratory-selected linezolid-resistant strain of *S. aureus* (Swaney *et al.,* 1998b). This finding was confirmed by Kloss *et al.,*(1999) who selected linezolid-resistant *Halobacterium halobium* (*H. halobium*) mutants, all of which had mutations within the central loop of domain V of their single 23S rRNA gene copy. This finding led the authors to propose that oxazolidinones inhibit the incorporation of $tRNA_f^{Met}$-ribosome-mRNA complex. Mutations in laboratory-selected linezolid-resistant *E. coli* isolates were also clustered in domain V (Xiong *et al.,* 2000). Bobkova *et al.,* (2003) proposed that oxazolidinones prevent binding of the initiator tRNA to the peptidyltransferase P site on the 50S subunit and hence the formation of the first peptide bond. It has been suggested that oxazolidinones may bind to a ribosomal protein and that mutations in the rRNA genes would therefore alter the protein conformation, affecting interactions between the drug and target (Kloss *et al.,* 1999). However, Kloss *et al.*, (1999) went on to suggest that if ribosomal proteins were directly involved in binding of oxazolidinones, resistance would arise at a greater frequency in these proteins. More recently, it was suggested that oxazolidinones bind to tRNA, proteins L27 and Lep and to nucleotides A2603 or G2608 (situated in the peptidyl transferase centre) of the genes encoding 23S rRNA (Colca *et al.,* 2003, Xu *et al.,* 2005). It was suggested that the $tRNA_f^{Met}$-ribosome-mRNA complex optimally exposes the ribosome to drug binding (Colca *et al.,* 2003, Xu *et al.,* 2005). Drug binding may "lock" the ribosome and prevent further conformational change (Colca *et al.,* 2003) or may block the exit tunnel between P and A sites (Xu *et al.,* 2005).

In short, whilst the evidence for binding of oxazolidinones in the peptidyltransferase region is conclusive, the exact binding site or sites remain elusive. Additionally, there is the possibility of interactions between oxazolidinones and ribosomal proteins.

1.3.3 Linezolid resistance

1.3.3.1 The emergence of resistance

It was thought that resistance to linezolid would be rare, firstly due to its unique mechanism of action, without known cross-resistance with any other classes of antibiotic and, secondly, due to gram-positive species having multiple copies of the 23S rRNA genes and spontaneous mutations being necessary in more than one of these gene copies before phenotypic resistance emerged. The difficulty in generating linezolid-resistant mutants of *S. aureus* and enterococci *in vitro* supported this hypothesis (Zurenko *et al.,* 1996). Nevertheless, the first linezolid-resistant mutants of *S. aureus* and *E. faecalis* were selected by Swaney *et al.*, (1998b) via a spiral gradient technique. Further reports of resistance in the laboratory followed (Table 4). In 1999, the first clinical linezolid-resistant strains were described (Zurenko *et al.,* 1999); resistance emerged in *Enterococcus faecium* (*E. faecium*) strains from two patients prescribed linezolid under the compassionate use programme. Resistance in clinical *S. aureus* isolate took slightly longer to emerge, but did so in the USA (Tsiodras *et al.,* 2001). Two years later the first clinical linezolid-resistant *S. aureus* was isolated in the UK (Wilson *et al.,* 2003). Reports of linezolid resistance in clinical isolates of CNS and streptococci have also emerged; the SENTRY and PROTEKT surveys reported linezolid-resistant *S. epidermidis*, *Streptococcus oralis* (*S. oralis*) and *S. pneumoniae* (Farrell *et al.,* 2004, Mutnick *et al.,* 2002). More recently, Fraimow *et al.*, (2005) and Potoski *et al.*, (2006) have reported linezolid-resistant *Staphylococcus lugdunensis*, *S. epidermidis* and *S. haemolyticus* and isolates from the clinic. Linezolid-resistant CNS from Ireland have also been reported to ARMRL (Neil Woodford, personal communication).

Resistance to linezolid in clinical strains of *S. aureus* is still infrequent, however, more occurrences are being reported (Gales *et al.,* 2006, Hill *et al.,* 2005, Machado *et al.,* 2003, Meka *et al.,* 2004a, Meka *et al.,* 2004b, Paterson *et al.,* 2003, Pillai *et al.,* 2002, Roberts *et al.,* 2006, Tsiodras *et al.,* 2001, Wilson *et al.,* 2003). In the majority of cases, linezolid resistance evolved during long-term therapy with linezolid. For example, early *E. faecium* isolates resistant to linezolid arose after 21 and 40 days treatment with linezolid (Gonzales *et al.,* 2001, Johnson *et al.,* 2002, Zurenko *et al.,* 1999). A linezolid-resistant *E. faecalis* was isolated after 25 days treatment

Table 4. Mutations conferring (gram-positive species) or increasing (*E. coli*) resistance to linezolid.

23S rRNA mutation	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Staphylococcus haemolyticus*	*Enterococcus faecium*	*Enterococcus faecalis*	*Streptococcus pneumoniae*	*Streptococcus oralis*	*Halobacterium halobium*	*Escherichia coli*	*Mycobacterium smegmatis*
G2032A									✓[13]	
G2057A						✓[17]				
A2059G						✓[17]				
A2062A								✓[22]		
C2118A						✓[18]				
T2132A							✓[15]			
A2160G						✓[19]				
C2192T	✓[1]									
T2211G				✓[9]			✓[15]			
T2312A				✓[9]						
T2357A				✓[9]			✓[15]			
G2445T	✓[2]									
G2447T/A	✓[3]								✓[14]	✓[23]
C2452T								✓[22]		
A2453G/C								✓[22]		
C2499T								✓[22]		
T2500A/C	✓[4] ✓[5]							✓[22]		

Cont.

Table 4. Mutations conferring (gram-positive species) or increasing (*E. coli*) resistance to linezolid (cont.).

23S rRNA mutation	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Staphylococcus haemolyticus*	*Enterococcus faecium*	*Enterococcus faecalis*	*Streptococcus pneumoniae*	*Streptococcus oralis*	*Halobacterium halobium*	*Escherichia coli*	*Mycobacterium smegmatis*
A2503G	✓[5]					✓[18]				
T2504C	✓[6]							✓[22]		
G2505A	✓[5]			✓[10]						
C2512T					✓[10]					
G2513T					✓[10]					
G2528T	✓[3]									
C2534T		✓[15]								
C2571G						✓[20]				
G2576T	✓[7] ✓[8]	✓[15]	✓[16]	✓[11]	✓[12] ✓[10]	✓[20]	✓[15]			
C2610G					✓[10]	✓[21]				
C2612A						✓[20]				

✓ Found in laboratory-selected mutant(s)

✓ Found in clinical isolate(s)

Superscript numbers refer to reference overleaf.

Cont.

1. **Howe *et al.***. 2002.

2. **Barrett**. 2000.

3. **Swaney *et al.***. 1998.

4. **Meka *et al.***. 2004.

5. **North *et al.***. 2005.

6. **North *et al.***. 2005.

7. **Tsiodras *et al.***. 2001.

8. **Bryskier**. 1999.

9. **Liao *et al.***. 2005.

10. **Prystowsky *et al.***. 2001.

11. **Zurenko *et al.***. 1999.

12. **Johnson *et al.***. 2002.

13. **Xiong *et al.***. 2000.

14. **Bobkova *et al.***. 2003.

15. **Mutnick *et al.***. 2002.

16. **Fraimow *et al.***. 2005.

17. **Farrell *et al.***. 2004.

18. **Carsenti *et al.***. 2003.

19. **Bozdogan *et al.***. 2002.

20. **Carsenti-Dellamonica *et al.***. 2005.

21. **Vandenbos *et al.***. 2003.

22. **Kloss *et al.***. 1999.

23. **Sander *et al.***. 2002.

(Johnson *et al.,* 2002), and three independent linezolid-resistant *S. aureus* isolates emerged after 21 days, one month and 20 months treatment, respectively (Meka *et al.,* 2004b, Tsiodras *et al.,* 2001, Wilson *et al.,* 2003). Long-term, low-dose treatment may facilitate selection of resistance (Hill *et al.,* 2005).

1.3.3.2 Mechanism of resistance

Sequencing of the 23S rRNA genes of the first mutants selected *in vitro* by Swaney *et al.*, (1998b) revealed a G to T change at position 2447 in *S. aureus* mutants and a G to T change at position 2528 in both *S. aureus* and *E. faecalis*. Subsequent clinical isolates of *S. aureus*, *E. faecium* and *E. faecalis* all had a G to T change at position 2576 (Johnson *et al.,* 2002, Tsiodras *et al.,* 2001, Zurenko *et al.,* 1999). Meka *et al.,* (2004b) characterized the only exception to this pattern; a clinical linezolid-resistant *S. aureus* with a T2500A mutation in three of its six 23S rRNA gene copies. Despite investigation, the reason for the repeated selection of the G2576T mutation in the clinic, independent of species, has not been determined (Lobritz *et al.,* 2003, Pillai *et al.,* 2002). Although this one mutation predominates clinically, a variety of mutations have been characterized in laboratory-selected mutants of several species (Table 4). Additionally, it is possible to generate the G2576T mutation in the laboratory (Bryskier, 1999). The location of these mutations, within the peptidyltransferase centre and in close proximity to A, P and E sites, serves to reinforce theories that linezolid binds to the 50S ribosomal subunit (Xiong *et al.,* 2000).

1.3.3.3 Genetic recombination of resistance

S. aureus typically has five or six 23S rRNA gene copies, *E. faecalis* has four and *E. faecium* has six (Klappenbach *et al.,* 2001). It was thought that a mutation conferring resistance to linezolid in one gene copy would be masked by the remaining wild-type gene copies and therefore that independent mutations would be required in at least two genes to convey phenotypic resistance (Willems *et al.,* 2003, Xiong *et al.,* 2000). The possibility of such spontaneous mutations occurring in two or more of the 23S rRNA genes was thought to be rare, however resistance still emerged as noted above. Lobritz *et al.*, (2003) proposed that a spontaneous mutation in one gene copy,

followed by homologous recombination events with wild-type copies enabled the proliferation of the mutation to other gene copies. This phenomenon has previously been implicated in increasing levels of resistance to aminoglycosides in *Mycobacterium smegmatis* (Prammananan *et al.,* 1999) and to evernimicin in *S. pneumoniae* (Adrian *et al.,* 2000). Experiments with *recA*-negative *E. faecalis* and *S. aureus* mutants confirmed that homologous recombination is the primary mechanism of spread of a mutation to multiple gene copies (Lobritz *et al.,* 2003, Miller *et al.,* 2005). Marshall *et al.*, (2002) proved a direct correlation between the linezolid MIC and the number of mutated gene copies in enterococci. So as long as a linezolid-resistant isolate retained at least one wild-type copy, reversion to susceptibility could occur via reversal of the same route. Meka *et al.*, (2004a) reported that in the continued absence of selection pressure, a linezolid-resistant *S. aureus* isolate from the clinic reverted to susceptibility. However, a linezolid-resistant mutant with all 23S rRNA gene copies mutated might not revert back to susceptibility via this mechanism and would need a spontaneous mutation at the original mutated base or wild-type DNA from a foreign source to revert to susceptibility, a finding confirmed by Pillai *et al.*, (Pillai *et al.,* 2002).

1.3.3.4 Other linezolid resistance mechanisms, not involving 23S rRNA

Although the main mechanism of resistance seems to be mutations in the peptidyltransferase region of the 23S rRNA genes, reports have emerged of cases where no mutation could be detected in this region in clinical isolates and laboratory mutants of staphylococci, enterococci and streptococci (Carsenti-Dellamonica *et al.,* 2005, Fraimow *et al.,* 2005, Prystowsky *et al.,* 2001). Furthermore, Wolter *et* al., (2005) found mutations in the ribosomal protein L4 of pneumococci with resistance to oxazolidinones, macrolides and chloramphenicol, and Farrell *et al.*, (2004) found a combination of 23S rRNA and L4 mutations conferred resistance to linezolid in a clinical *S. pneumoniae* isolate. There have been no reports to date of similar mutations in the ribosomal proteins conferring resistance to linezolid in staphylococci or enterococci. More recently a RNA methyltransferase, encoded by the *cfr* gene, has been shown to methylate position A2503 of the 23S rRNA genes, conferring resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A antibiotics in *E. coli* and *S. aureus* (Long *et al.,* 2006). This is the first

reported mechanism, so far, of linezolid resistance that is transferable. It has been detected in very few staphylococcal strains, although it is probably not widely looked for (Kehrenberg and Schwarz, 2006).

1.3.3.5 Unstable linezolid resistance

There have been reports of unstable linezolid resistance, especially when selected *in vitro*. Prystowsky *et al.*, (2001) found a two-fold reduction in the linezolid MIC (32 to 16 mg/L) for a resistant mutant of eneterococci after one month of growth on drug-free media. Similarly, Xiong *et al.*, (2000) used a laboratory strain of *E. coli* (growth was suppressed at high concentrations) to raise linezolid-resistant mutants. Only two of 12 *E. coli* mutants maintained a highly resistant phenotype after one passage in the absence of linezolid.

1.3.3.6 Cross-resistance between linezolid and other antibiotic classes

Although oxazolidinones are marketed as having no cross-resistance with any other class of antibiotic, studies designed to determine the binding site of linezolid, revealed partial cross-resistance between linezolid and other antibiotics when mechanisms other than G2576T are involved. Bobkova *et al.*, (2003) and Lin *et al.*, (1997) discovered that chloramphenicol and linezolid compete for binding sites, and that mutations G2032A and G2447A in the 23S rRNA genes resulted in reduced sensitivity to both these agents conferring partial cross-resistance. Likewise mutations at positions 2062 and 2452 in laboratory-selected linezolid-resistant mutants of *Halobacterium halobium* conferred resistance to both linezolid and chloramphenicol (Kloss *et al.*, 1999, Mankin and Garrett, 1991). In this context, some of the nucleotides that chloramphenicol interacts with most strongly have also been implicated in linezolid resistance, e.g., 2061, 2452, 2500, 2504, 2505, 2506 (Table 4) (Schlunzen *et al.*, 2001).

Howe *et al.*, (2002) first noticed that the emergence of linezolid resistance was associated with loss of erythromycin resistance in laboratory-selected mutants of *S. aureus*. Wilson *et al.*, (2003) confirmed this finding for a clinical isolate of *S. aureus*, and the relationship was also found in *S. pneumoniae* mutants (Carsenti *et al.*, 2003). However, the emergence of linezolid resistance does

not always result in the loss of erythromycin resistance (Meka *et al.,* 2004b). To determine whether linezolid resistance and the G2576T mutation were incompatible with erythromycin resistance Sakoulas *et al.*, (2003) tried to restore susceptibility in a linezolid-resistant *S. aureus* strain by introducing an *erm*(C) determinant. The successful incorporation of *erm*(C) did not result in linezolid susceptibility. It is not known why linezolid resistance causes loss of erythromycin determinants in some strains.

1.3.3.7 Detection of the molecular mechanism of linezolid resistance

Initial identification of a linezolid resistance mechanism was made by amplifying a region of the 23S rRNA genes, followed by DNA sequencing (Swaney *et al.,* 1998b). This enabled the detection of single nucleotide polymorphisms and gave a rough indication as to the ratios of wild-type versus mutated 23S rRNA gene copies (Swaney *et al.,* 1998b). The realization that most linezolid-resistant clinical isolates had the same mutation, G2576T, led to the development of a more rapid detection assay. Amplification of the 23S rRNA genes, followed by digestion with a restriction endonuclease *Nhe*I enabled detection of the G2576T mutation in staphylococci and enterococci. Furthermore, the restriction pattern produced allowed the distinction between hetero- and homozygosity (Woodford *et al.,* 2002). This method was adapted to allow a degree of gene quantitation by densometrically measuring the amounts of digested and undigested amplicons (Mazur *et al.,* 2002). The same principle has been applied to detect other mutations thought to confer linezolid resistance. Digestion of an amplified fragment of the 23S rRNA genes with *Eco*RV will detect a G2505A mutation and with *Hin*1I will detect a T2504C mutation (Lobritz *et al.,* 2003, North *et al.,* 2005b). Hetero- and homozygosity can be determined in both cases. This technique is accurate and can be applied to detect a variety of mutations, so long as these result in the acquisition or loss of a restriction site. Real-time PCR, with a lightcycler or Taqman system, was found to be a fast and accurate technique for the identification of a G2576T mutation in enterococci, although the need for expensive equipment is a disadvantage (Werner *et al.,* 2004, Woodford *et al.,* 2002).

Pyrosequencing®[A] was successfully used for the detection of a G2576T mutation and estimation of the number of mutated 23S rRNA gene copies in enterococci (Sinclair *et al.,* 2003). During the pyrosequencing reaction, a sequencing primer hybridizes to single-stranded template DNA. The successful incorporation of a complementary nucleotide results in the emission of light that can be detected. The amount of light generated is directly proportional to the amount of nucleotide incorporated, thereby allowing quantification. However, this method works on the assumption that samples of the same species have a set amount of gene copies (Sinclair *et al.,* 2003). In order to determine the number of 23S rRNA gene copies in linezolid-resistant isolates, Pillai *et al.*, (2002) digested genomic DNA with the restriction endonuclease *Eco*RI. There is an identical *Eco*RI restriction site in each of the 23S rRNA genes and an additional variable restriction site downstream of each gene, meaning each gene can be distinguished by size. The products of digestion were separated by gel electrophoresis and southern blotted. Hybridization with a 420 bp DIG (digoxigenin)-labelled probe corresponding to domain V of the 23S rRNA genes then enabled detection of each of the gene copies. Furthermore, by digesting genomic DNA with both *Nhe*I and *Eco*RI 23S rRNA gene copies with a G2576T mutation were detectable. This technique allows successful quantification of 23S rRNA gene copies, additionally, it allows comparison of restriction profiles produced by different strains. However, this method assumes that the downstream *Eco*RI sites are in different locations in all gene copies. Identical restriction sites in two or more copies would not be detectable. A similar technique was employed by Marshall *et al.*, (2002) who used the restriction endonucleases *Eco*RI and *Mae*I to determine, firstly the number of 23S rRNA gene copies and secondly, which of those had G2576T mutations.

1.3.3.8 Nosocomial spread of linezolid resistance

Resistance to linezolid, although still rare, is increasing, possibly due to growing use (Hogan *et al.,* 2005). Resistance is confined to individual patient isolates in most cases, although in 2002 and 2003, nosocomial spread of linezolid-resistant *E. faecium* strains were reported in the USA (Engemann *et al.,* 2003, Herrero *et al.,* 2002). More recently, reports of nosocomial spread of

([A] Pyrosequencing and Pyrogram are registered trademarks of Biotage).

linezolid-resistant CNS in the USA have been published (Fraimow *et al.*, 2005, Potoski *et al.*, 2006). Since the horizontal transfer of resistance is unlikely to occur, early detection would prevent the spread of such linezolid-resistant strains between patients.

1.4 Hypermutability

One concern for the emergence of linezolid resistance is the presence of strains with an elevated mutation rate which might be predisposed to developing resistance to linezolid. Hypermutability is a term used to describe the situation of organisms with elevated mutation rates. The mechanisms underlying hypermutability are most often associated with mutations in the genes encoding the DNA mismatch repair (MMR) machinery, resulting in the failure to correct mismatched bases incorporated during replication. In turn, this results in a hypermutable phenotype. Strains in the clinic with a hypermutable phenotype could be pre-disposed to developing resistance to antibiotics, especially where resistance is conferred by a single chromosomal mutation, followed by recombination, as is the case with linezolid. This section discusses the processes involved in DNA repair and the evidence for hypermutability in bacterial populations.

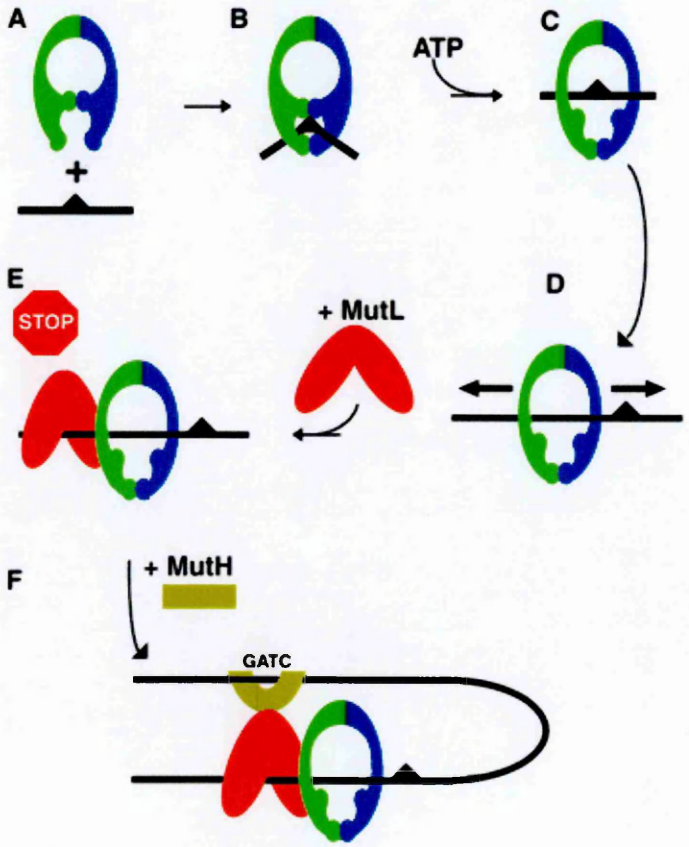
1.4.1 DNA repair mechanisms

1.4.1.1 Mismatch repair

Mutations in DNA arise during replication, homologous recombination, and as a result of DNA damage. Bacteria have DNA repair mechanisms in place to correct these mutations and ensure the fidelity of the genome; one of these is the MMR pathway (Schofield and Hsieh, 2003).

The MMR machinery is thought to be able to distinguish between the hemimethylated parent and daughter DNA strands and can direct its component parts, MutS, MutL and MutH, towards the daughter strand (Yang, 2000). MutS proteins follow behind the DNA polymerase and scan the newly synthesised strand, recognising and binding to seven of eight possible mismatches as well as to short insertions or deletions that have arisen via misincorporation of nucleotides or template slippage (Figure 6) (Schofield *et al.*, 2001). MutS, present as a homodimer, recognises and binds

Figure 6. The interaction of MutS and MutL at a mismatch. (From Schofield *et al.*, (2001). MutS in solution **(A)** binds to a mismatch in an induced fit model involving extensive conformational changes in both MutS and the mismatch DNA **(B)**. Binding of ATP induces an additional conformation change **(C)** resulting in diffusion of MutS away from the mismatch **(D)**. The signal to initiate downstream events is provided when MutL associates with MutS that has bound both a mismatch and ATP and prevents MutS from leaving the immediate vicinity of the mismatch **(E)**. This MutS-MutL complex is postulated to be a critical intermediate in the activation of MutH for incision of the daughter strand **(F)**.



to a mismatch, capturing the DNA between the two MutS molecules (Lamers *et al.,* 2000). The binding of a DNA mismatch and ATP produces a conformational change in the MutS homodimer (Lamers *et al.,* 2000, Lamers *et al.,* 2004, Schofield *et al.,* 2001). There are several theories as to how the binding of MutS to a mismatch signals subsequent proteins to assemble at the site of the mismatch. Allen *et al.*, (1997) and Blackwell *et al.*, (1998) proposed the translocation of MutS away from the mismatch was the trigger. Gradia *et al.*, (1997, 1999) suggested the transformation of MutS into a sliding clamp which diffuses away from the mismatch triggered downstream events. Alternatively Junop *et al.*, (2001) and Schofield *et al.*, (2001) proposed the association of MutL with MutS that has bound ATP *in situ* was the trigger for ensuing actions. Regardless of the exact mechanism, MutL and MutH assemble at the site of the mismatch. MutL associates with MutS and the mismatch, and is thought to activate MutH and mediate interactions between MutS and MutH (Grilley *et al.,* 1989). Additionally Schofield *et al.*, (2001) proposed that MutL prevents MutS from leaving the immediate vicinity of the mismatch. In *E. coli* MutH is an endonuclease that nicks the daughter strand at methylation sites (GATC), enabling strand discrimination. However, no MutH protein has been found in gram-positive bacteria nor in some species of gram-negative bacteria, including *Neisseria* and *Pseudomonas aeruginosa* (*P. aeruginosa*); rather it has been suggested an endonuclease homologue breaks the newly synthesised strand enabling strand discrimination (Jacquelin *et al.,* 2005, Kline *et al.,* 2003, Merino *et al.,* 2002). Next, DNA helicase separates the two DNA strands, the defective daughter strand is degraded by an exonuclease and DNA polymerase fills in the gap before DNA ligase seals the strands (Yang, 2000).

The mismatch repair system is also thought to police homologous recombination events to ensure the fidelity of the genome. It has been shown to act as a barrier to interspecies recombination between *E. coli* and *Salmonella.* Inactivation of this system enables the transduction (i.e., phage mediated transfer) of chromosomal genes between similar species (Matic *et al.,* 1994, Zahrt *et al.,* 1994).

1.4.1.2 SOS response

Another DNA repair mechanism implicated in hypermutability is the SOS response. In 1974 Radman determined that when bacteria are subjected to stress, proteins are produced that repair

DNA damage and reactivate DNA synthesis. He proposed that this process, termed the SOS response, was also linked to mutability (Radman, 1974). The SOS response is controlled by two genes: *lexA* encodes for a suppressor protein and *recA* encodes for an anti-repressor protein, required to remove LexA (Gudas and Pardee, 1975). The SOS genes are scattered around the chromosome and their expression is controlled by the proteins LexA and RecA. LexA binds to the operons of the SOS genes and represses their transcription. When DNA is damaged, RecA facilitates the cleavage of LexA, decreasing the levels of LexA and enabling expression of the SOS genes (Little and Mount, 1982). In *E. coli*, three error-prone DNA polymerases are induced (Pol II, Pol IV and Pol V) as part of the SOS response (Yeiser *et al.,* 2002). Their induction and involvement in DNA repair results in an increase in the number of mutations within the genome and, hence, a competitive advantage (Yeiser *et al.,* 2002).

1.4.2 Hypermutability in clinical isolates

Hypermutators have been found in naturally occurring bacterial populations. LeClerc *et al.*, (1996) determined that hypermutable isolates comprised more than 1% of *E. coli* and salmonella pathogens. Matic *et al.*, (1997) surveyed mutation frequencies of *E. coli* isolates from different environments and discovered a high incidence of mutators, both pathogenic and commensal.

Hypermutators have also been associated with clinical bacterial populations, especially in the lungs of patients with cystic fibrosis. Cystic fibrosis sufferers have altered electrolyte secretion by cells in the lungs, resulting in the production of a viscous mucus. Consequently the lung's major anti-infective defence systems are impaired, resulting in chronic lung infection (Prunier *et al.,* 2003). The most commonly isolated species from the sputum of cystic fibrosis patients are *P. aeruginosa* (80%) and *S. aureus* (33-60%) (Govan and Nelson, 1992). Repeated treatment with antibiotics to combat persistent infections selects for multiply-resistant strains (Macia *et al.,* 2005).

Oliver *et al.*, (2000) determined mutation frequencies to rifampicin for *P. aeruginosa* strains from 30 cystic fibrosis patients. Results revealed 37% of patients harboured strains with a mutator phenotype whereas no hypermutable strains were isolated from non-cystic fibrosis patients. The epidemiology of these mutator strains suggested one or two successful strains that persisted in the

lungs of patients over a number of years. Deletions in the *mutS* gene were detected in some strains and complementation of *mutS* lowered their mutation frequencies. A similar finding was made by Maciá *et al.*, (2005), who concluded that hypermutable strains of *P. aeruginosa* were extremely frequent in cystic fibrosis patients. However, a conflicting report from Gutiérrez *et al.*, (2004) concluded that the prevalence of hypermutable strains of *P. aeruginosa* in cystic fibrosis patients was low. In other species, Roman *et al.*, (2004) reported hypermutable strains of *Haemophilus influenzae* (*H. influenzae*) in 33% of cystic fibrosis patients and Watson *et al.*, (2004) found hypermutable *H. influenzae* isolates to have polymorphisms in the *mutS* gene.

Less research has been conducted concerning the prevalence of hypermutable *S. aureus* strains in cystic fibrosis patients. Prunier *et al*, (2003) found 13 of 89 isolates from cystic fibrosis patients were hypermutable and alterations in the *mutS* gene were found in five of eleven (45%) of these hypermutable strains tested. Conversely, O'Neill *et al*., (2002) studied 49 *S. aureus* isolates from cystic fibrosis patients and found little evidence for the existence of hypermutability.

It should be cautioned that comparisons between studies are difficult due to the lack of a standardized definition of hypermutability. Studies refer to hypermutable isolates as displaying mutation frequencies greater than 10^{-7} (Prunier *et al.,* 2003) or $\geq$ 10-fold greater than a laboratory strain (O'Neill and Chopra, 2002); alternatively divisions between mutators and non-mutators are made on a relative basis according to the results obtained in the study (Oliver *et al.,* 2000). Differences in methodologies could also be a source of varying mutation frequencies.

1.4.3 Transient hypermutability

Hypermutable organisms are not always detectable within multi-resistant bacterial populations. This is possibly because the organisms pass through temporary periods of high mutation, enabling rapid generation of genetic diversity to occur, followed by restoration of lower mutation rates. Perhaps such a period of transient hypermutability would reduce the risk of deleterious mutations that could have a fitness cost or lethal consequences (Chopra *et al.,* 2003, Denamur *et al.,* 2002). Alternatively it is possible that, within a bacterial population, a proportion of organisms are always undergoing a period of hypermutability, ensuring the population can benefit as a whole, if and

when necessary, without suffering disadvantageous mutations (Blazquez *et al.,* 2002). However, proving that transient periods of hypermutability occur is difficult, as previous episodes do not leave detectable markers. Denamur *et al.*, (2002) suggested that *E. coli* strains repeatedly pass through periods of high mutations rates during their evolutionary history, regardless of whether they are commensal or pathogenic.

These periods of hypermutability could be stress induced. Bjedov *et al.*, (2003) found more than 50% of worldwide strains of *E. coli* showed a high rate of stress induced (carbon-source starvation and oxidative shock) mutagenesis as a result of down-regulation of the MMR system and up-regulation of the SOS response. Furthermore, Denamur *et al.*, (2000) proposed that MMR genes passed through periods of high mutation and recombination, allowing rapid generation of genetic diversity and adaptation, followed by a means of restoring lower mutation rates.

The induction of the SOS response is probably the best known example of transient hypermutability. DNA damage results in the induction of the SOS system, an error-prone DNA repair pathway, which results in many mutations. A selected beneficial mutation will enable survival and result in the down-regulation of the SOS response.

1.4.4 Hypermutability and the emergence of antibiotic resistance

Organisms containing a beneficial antibiotic resistance mechanism will be selected in the presence of that antibiotic. Furthermore, it has been suggested that antibiotics can inadvertently increase the chances of resistance occurring to new antimicrobials by selecting for mutator strains (Blazquez *et al.,* 2002, Blazquez, 2003, Livermore, 2003). This theory assumes, to a certain extent, that hypermutability may be co-selected with resistance, since resistance is more likely to evolve in hypermutable strains but direct evidence for this theory is conflicting.

Mao *et al.*, (1997) proposed that a single selection step can increase the percentage of mutators from 0.001% to 0.5% in a strain of *E. coli* and subsequent selection steps can result in the majority of the population being over-run by mutators. Komp *et al.*, (2003), after studying *E. coli* isolates from urinary tract infections, determined that there was a strong relationship between antibiotic resistance and high mutation rate; selection for mutations conferring fluoroquinolone resistance co-

selected for strains with an elevated mutation rate. Similarly, Miller *et al.*, (2004b) proposed that the presence of mutators offered an enhanced risk for the emergence of antibiotic resistance during treatment of urinary tract infections (UTI) infections. However, Denamur *et al.*, (2002) did not find mutator strains to be more often resistant than non-mutators and concluded that antibiotic selection pressure was probably not the major factor in the emergence of mutator strains. Baquero *et al.*, (2004) likewise were unable to find any significant association between mutator phenotypes and antibiotic resistance in *E. coli* isolates from the clinic and from healthy volunteers from Spain, Denmark and Sweden.

Several of the studies involving isolates from cystic fibrosis patients make the association between hypermutability and the emergence of mutational antibiotic resistance. Oliver *et al.*, (2000) hypothesized that rapid adaptation was required for survival in the lungs of patients with cystic fibrosis. Mutator *P. aeruginosa* strains were found to have multiple resistances, and due to their status as hypermutators, were likely to become resistant to new compounds. Prunier *et al.*, (2003) investigated the mechanisms of resistance to macrolides in hypermutable *S. aureus* strains from cystic fibrosis patients and concluded that ribosomal mutation was the prevalent mechanism of resistance, possibly due to the higher proportion of hypermutable strains present. Furthermore, Giraud *et al.*, (2002) concluded that by selecting for a resistance allele, a mutator allele was also selected as the mechanism that generated the resistance, therefore one failed therapy is a potential risk factor for the next. Oliver *et al.*, (2004) and Negri *et al.*, (2002) have also suggested the use of low, sub-inhibitory concentrations of antibiotics could provide conditions for the selection of hypermutable strains.

It has been suggested that certain DNA damaging antibiotics, such as fluoroquinolones, cause an increase in the mutation rate by inducing the SOS response (Phillips *et al.*, 1987). Somewhat ironically, this induction of the SOS response could initiate a high mutation rate and therefore a greater chance of the emergence of favourable mutations that could result in resistance to the inducing antibiotic. Resistance to quinolones is associated with several mutations and the accrual of multiple mutations is associated with a corresponding increase in MIC (Ferrero *et al.*, 1995, Fournier and Hooper, 1998, Ito *et al.*, 1994). It was thought that the requirement of multiple mutations may delay the emergence of resistance to quinolones, however, hypermutators may

rapidly provide organisms with the necessary mutations (Wang *et al.*, 1998). This induction of the SOS response is not solely limited to DNA damaging antibiotics; more recently Miller *et al.*, (2004a) and Maiques *et al.*, (2006) discovered that β-lactam antibiotics are an extracellular stimulator of the SOS response in *E. coli* and *S. aureus*. By using SOS-inducible prophages, Maiques *et al.*, (2006) showed exposure to β-lactam antibiotics resulted in phage reproduction and therefore induction of the SOS response in *S. aureus*. They also confirmed the finding of Ubeda *et al.*, (2005) who noted exposure to β-lactam antibiotics promoted the transfer of virulence factors via pathogenicity islands in an SOS-dependent manner in *S. aureus* (Maiques *et al.*, 2006).

Persistent exposure to antibiotics selects for antibiotic-resistant strains. The question is whether, in selecting for antibiotic-resistant strains, hypermutable strains are being co-selected, resulting in a population with a high number of mutators and therefore a more favourable chance of surviving the next antibiotic exposure? These hypermutators could present an increased risk of resistance occurring to antibiotics where resistance is conferred by chromosomal mutation, such as linezolid. Therefore treatment with multiple antibiotics is advisable (Chopra *et al.*, 2003, Giraud *et al.*, 2002, Oliver *et al.*, 2004). Although the evidence is not entirely conclusive, this could explain the presence of hypermutable multi-resistant strains in cystic fibrosis patients and those with persistent UTI infections. Conflicting evidence does leave the door open for other unknown mechanisms for mutator selection.

1.5 Summary

In summary, *S. aureus* is a major nosocomial pathogen, worldwide, which is now emerging in the community setting. Many *S. aureus* strains are resistant to many antibiotics, meaning few treatment options are available. Linezolid, along with teicoplanin and vancomycin, is one of the antibiotics of last resort available to treat multi-resistant *S. aureus* strains. Resistance to linezolid in clinical isolates of *S. aureus* is rare but, nevertheless, does occur. Similarly, chromosomal mutational resistance to teicoplanin and vancomycin has been detected in *S. aureus* isolates from the clinical setting. It has been proposed that the emergence of chromosomal mutations conferring resistance to these antibiotics might be facilitated by a hypermutable phenotype. Due to a faulty

mismatch repair system, a higher proportion of chromosomal mutations go uncorrected. This in turn might accelerate the development of resistance in the population and might lead to the co-selection of hypermutability with these difficult to select resistances.

1.6 Aims and hypothesis

At the beginning of this project (March 2003) resistance to linezolid was infrequent, however, it had been reported in clinical isolates of *E. faecium* and *E. faecalis* (Zurenko *et al.*, 1999) and in one clinical isolate of *S. aureus* (Tsiodras *et al.*, 2001). Preliminary studies indicated that resistance was due to single nucleotide polymorphisms in the 23S rRNA genes. At this time the involvement of hypermutability in the emergence of linezolid resistance was unknown, although the importance of hypermutability in general was increasingly discussed, mostly in connection with gram-negative bacteria. Reports on the presence of hypermutable *S. aureus* strains in the clinic were limited. Therefore the aims of this project were:

(i) to study the mechanism and development of resistance to linezolid in staphylococci, including assessing the number of 23S rRNA copies mutated in resistant isolates and mutants

(ii) to investigate the role of hypermutability in the emergence of linezolid and teicoplanin resistance

(iii) to establish whether teicoplanin-resistant isolates express a hypermutator phenotype and as a consequence have an increased capacity to develop resistance to other anti-gram-positive antibiotics, such as linezolid.

The hypothesis to be tested was that resistance to linezolid could emerge more readily in hypermutable strains of *S. aureus*, and that hypermutability might be co-selected with resistance to linezolid or glycopeptide antibiotics.

2 Materials and methods

2.1 Bacterial identification

2.1.1 Clinical isolates

S. aureus isolates, obtained from hospital patients in the UK or Brazil (Table 5) and suspected to have reduced susceptibility to linezolid, were referred to the Antibiotic Resistance Monitoring and Reference Laboratory (ARMRL) at the Centre for Infections, Health Protection Agency (HPA) (London) for testing. A representative MSSA isolate, from the clinic, was used when establishing PFGE profiles and mutation frequencies. A pair of genetically-related teicoplanin-susceptible and -resistant isolates (ST/03/2121 and ST/03/2122), where resistance to teicoplanin emerged during therapy, were used for the generation of linezolid-resistant mutants and estimation of mutation frequencies. Genetically-related erythromycin-susceptible and -resistant pairs of *S. aureus* isolates, referred to ARMRL in 2002, were used to select linezolid-resistant mutants *in-vitro*. Details of these strains are listed in Table 6. A clinical *S. aureus* isolate, ST 251, was used as an internal control strain when determining MICs (Table 7). Teicoplanin-resistant *S. aureus* isolates, submitted to ARMRL for antibiotic testing from 2003-2005, were used to determine mutation frequencies to rifampicin and fusidic acid (Table 8).

2.1.2 Reference strains

Reference strains of *S. aureus* ATCC 29213, NCTC 12493 and NCTC 6571, were used as controls when determining MICs (Table 7). MICs of a range of antibiotics for these strains are shown in Table 7. Strains NCTC 13142 and NCTC 13143 were used as representatives of EMRSA-15 and EMRSA-16, respectively (Table 6). The laboratory strain RN4220 was used as a wild-type control strain and its *mutS*-negative mutant, RN4220Δ*mutS*, as a hypermutable control strain (O'Neill and Chopra, 2002) (Table 6).

Table 5. Suspected linezolid-resistant clinical isolates of *S. aureus* used in this study.

Patient No.	Isolate	Place of isolation	Site of isolation
1a	H045360367	Kent (UK)	Sputum (CF[A] patient)
1b	H053540293	Kent (UK)	Sputum (CF patient)
2a	H045360368	Kent (UK)	Sputum (CF patient)
2b	H053540294	Kent (UK)	Sputum (CF patient)
3a	H042800236	Nottingham (UK)	Swab from penis
3b	H042800257	Nottingham (UK)	Swab from drain site
3c	H042800258	Nottingham (UK)	Swab from perineum
4a	H053760467	Dublin (UK)	Sputum (CF patient)
4b	H053760478	Dublin (UK)	Sputum (CF patient)
5a	Brazilian 1 (B1)	Porto Alegre (Brazil)	Sputum (CF patient)
5b	Brazilian 2 (B2)	Porto Alegre (Brazil)	Sputum (CF patient)

[A] Cystic fibrosis (CF)

Table 6. Control strains of *S. aureus*.

Isolate	Clinical/Lab strain	Origin (UK)	Strain	Reference
H051000333	Clinical strain	Essex	MSSA	This study
ST/03/2121	Clinical strain	Leeds	EMRSA-15	This study[A]
ST/03/2122	Clinical strain	Leeds	EMRSA-15	This study[A]
7499	Clinical strain	Nottingham	EMRSA-15	This study
7500	Clinical strain	Nottingham	EMRSA-15	This study
7717	Clinical strain	Nottingham	EMRSA-15	This study
7501	Clinical strain	Nottingham	EMRSA-15	This study
ST 251	Clinical strain	London	MSSA	This study
ATCC 29213	Laboratory strain	N/A	MSSA	(Andrews, 2001)
NCTC 12493	Laboratory strain	N/A	MRSA	(Andrews, 2001)
NCTC 6571	Laboratory strain	N/A	MSSA	(Andrews, 2001)
NCTC 13142	Laboratory strain	N/A	EMRSA-15	(O'Neill *et al.*, 2001)
NCTC 13143	Laboratory strain	N/A	EMRSA-16	(Murchan *et al.*, 2004)
RN4220	Laboratory strain	Leeds	MSSA	(O'Neill and Chopra, 2002)
RN4220Δ*mutS*	Laboratory strain	Leeds	MSSA	(O'Neill and Chopra, 2002)[B]

[A] Related pair, differing in teicoplanin resistance

[B] Hypermutable

Table 7. MICs (mg/L) of relevant antibiotics for control strains.

Antibiotic	BSAC breakpoints[A]	ATCC 29213	NCTC 12493	NCTC 6571	ST 251
Chloramphenicol	8	8	8	2	8
Ciprofloxacin	1	0.5	2	0.12	1
Clindamycin	0.5	≤0.25	>8	0.06	≤0.25
Erythromycin	0.5	≤0.25	>16	0.12	>16
Fusidic acid	1	0.5	≤0.25	0.06	0.5
Linezolid	4	2	1	0.5	2
Oxacillin	4	≤0.25	8	N/A	4
Quinupristin/dalfopristin	2	0.5	0.5	0.12	0.5
Rifampicin	0.06	≤0.03	≤0.008	0.004	0.06
Teicoplanin	4	0.5	≤1	0.25	1
Vancomycin	4	0.5	2	0.5	1
Fosfomycin	N/A	N/A	N/A	N/A	N/A

[A] According to the BSAC guidelines (Andrews, 2001, Andrews, 2005).

Table 8. Teicoplanin-resistant *S. aureus* clinical isolates.

Patient No.	Isolate	Place of isolation (UK)	Site of isolation	Teicoplanin MIC (mg/L)
6	H034840069	Surrey	Skin infection	16
7	H035220342	London	Blood infection	8
8	H041340156	Surrey	Not provided	16
9	H041560345	Hampshire	Skin infection	16
10	H041560346	Hampshire	Skin infection	16
11	H041560348	Hampshire	Skin infection	16
12	H042240308	Gloucestershire	Septicaemia	8
13	H043100413	London	Chest infection	8
14	H043740188	London	Blood infection	8
15	H044640520	Middlesex	Skin infection	8
16	H044640521	Middlesex	Sputum	8
17	H044920446	Middlesex	Drain site	8
18	H045000304	Middlesex	Wound site	8

2.1.3 Identification of *S. aureus*

Isolates submitted to the Staphylococcus Reference Unit (HPA, London) for testing, presumed to be *S. aureus*, were identified by plating on CHROMagar (agar 15.0 g/L, peptone 40.0 g/L, sodium chloride 25.0 g/L, chromogenic mix 2.5 g/L, pH 6.9). *S. aureus* colonies appeared mauve, while other bacteria appeared blue, white or growth was inhibited (e.g. *S. haemolyticus* appeared blue).

2.2 Media, chemicals and apparatus

2.2.1 Media

Brain Heart Infusion (BHI) agar and broth, IsoSensitest (ISO) agar and broth, Nutrient Agar (NA) and Nutrient Broth (NB), Mueller-Hinton Agar (MHA), Columbia Agar (CA), Columbia Blood Agar (CBA) and Phosphate-Buffered Saline (PBS) (0.01 M, pH 7.4), (all from Oxoid Ltd. [Basingstoke, UK]) and CHROMagar (CHROMagar Microbiology, Paris) were used during the course of this work. See Appendix A Table 40 for more details.

2.2.2 Chemicals and apparatus

Chemicals and apparatus used in this study, together with a list of suppliers, are shown in Appendix A Table 41 and Table 42.

2.3 Antibiotic susceptibility testing

2.3.1 Antibiotics

The following antibiotics were used: chloramphenicol, ciprofloxacin, clindamycin, erythromycin, fosfomycin, fusidic acid, linezolid, oxacillin, quinupristin/dalfopristin, rifampicin, teicoplanin and vancomycin (see Appendix A Table 43 for suppliers). Antibiotics were dissolved according to BSAC guidelines (Andrews, 2001, Andrews, 2005). A stock solution was prepared and diluted,

with an appropriate diluent, to give the required antibiotic range after being incorporated into agar (MacGowan and Wise, 2001). See Table 7 for antibiotic ranges tested and breakpoints used.

2.3.2 Determination of MICs by agar dilution

MICs were determined according to BSAC guidelines (Andrews, 2001, Andrews, 2005). Freshly-grown bacteria were suspended to a density of a 1.0 McFarland standard (approximately 1.0×10^8 colony forming units (cfu/ml) in NB to ensure the correct inoculation density. Control strains with known MICs were included (Table 6 and Table 7). Antibiotics were incorporated into a series of ISO agar plates to yield a two-fold dilution series. ISO agar control plates, with no antibiotic incorporated, were also poured. Oxacillin was incorporated into CA with the addition of 2% NaCl. Where fosfomycin was incorporated into agar, 1 mg/L G-6-P was added to molten agar before pouring; G-6-P induces the glycerophosphate transport system, which aids the transport of fosfomycin into the cell (Kahan *et al.,* 1974). Plates were dried before being inoculated with 10^4 cfu/spot, using a multipoint inoculator, and were incubated at 37°C for 18-20 hours, except for oxacillin, which was incubated at 30°C for 24 hours. After incubation, control plates were checked for growth and MICs were read as the lowest concentration of antibiotic at which there was no visible growth of the organism. MICs for control organisms were checked to ensure they were within one two-fold dilution of the expected MIC (Table 7).

2.3.3 Determination of MICs by E Test

E Test strips were used to determine MICs of chloramphenicol, erythromycin, fosfomycin, fusidic acid, linezolid, rifampicin, teicoplanin and vancomycin. Freshly-grown bacteria were suspended to a density of a 1.0 McFarland standard in NB and used to inoculate an ISO agar plate with a swab so as to yield confluent growth. An E Test strip was placed on the agar in the direction of inoculation. The plate was incubated for 18-20 hours. The MIC was read at the point where the area of growth inhibition (including haze and isolated colonies) intersected the scale. The end point was read at complete inhibition of all growth, except for linezolid, which was read at 80% inhibition.

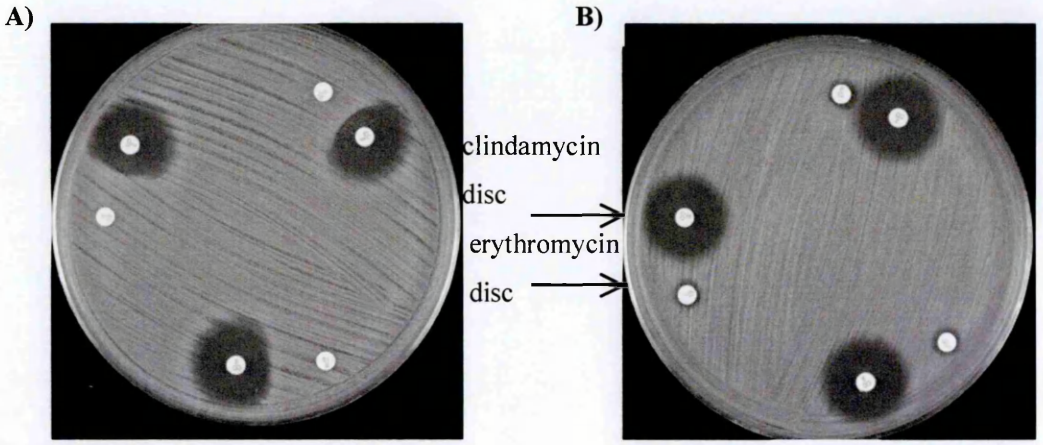
2.3.4 Population analysis

Population analysis was used to identify heterogeneous resistance to vancomycin and teicoplanin by detecting the existence of a sub-population of organisms for which the MIC was above the breakpoint. A streaked plate of freshly-grown bacteria was used to inoculate 10 mls of ISO broth which was incubated at 37°C for 24 hours. BHI agar plates containing appropriate dilutions of antibiotic (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mg/L) were poured and dried. Serial dilutions of 10^{-1} to 10^{-8} were prepared in PBS, from the overnight growth, and 2 x 20 µl drops of each dilution where plated at each antibiotic concentration and, additionally, on antibiotic-free media (CBA). Plates were incubated at 37°C for 24 hours. The viable count on antibiotic-free agar and at each antibiotic concentration was calculated and plotted.

2.3.5 Erythromycin/clindamycin synergy test

In order to detect inducible or constitutive macrolide resistance encoded by *erm* genes, a 0.5 McFarland standard suspension was prepared from freshly-grown bacteria and spread on MHA, so as to yield confluent growth. Erythromycin discs (15 µg) and clindamycin discs (2 µg) were placed 15-20 mm apart. Following incubation at 37°C for 16-18 hours, growth up to the edge of the clindamycin disc was considered to indicate constitutive resistance while any flattening or blunting of the clindamycin zone was considered to indicate inducible resistance (Figure 7) (Fiebelkorn *et al.*, 2003). It is acknowledged that growth up to the edge of the clindamycin disc could also indicate the presence of a lincosamide resistance mechanism, such as *lin*(A) along with an independent mechanism of macrolide resistance, e.g., by efflux (Lina *et al.*, 1999b), but these combinations are rare.

Figure 7. Disc diffusion testing for inducible clindamycin resistance in *S. aureus* (From Fiebelkorn *et al.*, 2003). **(A)** Positive disc induction test showing blunting of the zones around the clindamycin disc, indicating inducible lincosamide resistance. **(B)** Negative disc induction test indicating the absence of inducible lincosamide resistance.



2.4 Growth of *S. aureus*

2.4.1 Growth conditions

RN4220Δ*mutS* was grown at 42°C or in the presence of a 30 μg erythromycin disc to maintain integration of the thermosensitive plasmid used to disrupt the *mutS* gene. All other strains were grown at 37°C.

2.4.2 Replica plating

Freshly grown bacteria were suspended to a density of a 1.0 McFarland standard in NB which was then serially diluted from 10^{-1} to 10^{-8} in PBS. Aliquots (200 μl) of 10^{-4} to 10^{-8} dilutions were spread on BHI agar plates and incubated at 37°C for 24 hours. Plates with between 50 and 100 colonies were selected and colonies were transferred to BHI agar plates, one containing a suitable antibiotic, and the other drug-free, by replica plating, using a circular wooden block covered with sterile velvet. The two replica plates were then incubated for a further 24 hours at 37°C. The number of colonies on the original and both replica plates were recorded and compared.

2.4.3 Growth curves

Approximately four colonies of freshly-grown bacteria were used to inoculate 5 mls of ISO broth which then was incubated overnight at 37°C. ISO broth, 50 ml, was inoculated to a starting density of 0.05-0.1 (as measured at 600 nm) using approximately 100-200 μl of this overnight broth. This culture was incubated at 37°C, with gentle shaking, for approximately 9-10 hours. Samples were taken and the density read at 600 nm on a spectrophotometer every 10 minutes for the first 5 hours and every 45 minutes thereafter. To test whether the growth of erythromycin-resistant strains were affected by the presence of erythromycin, 100 mg/L of this drug was added to the broths.

2.5 Selection of antibiotic-resistant *S. aureus* mutants *in vitro*

2.5.1 Selection of linezolid-resistant mutants

2.5.1.1 Method A

Strains were grown in 10 ml ISO broth for 24 hours. One millilitre of this culture was transferred to 10 ml fresh ISO broth and incubated at 37°C for approximately seven hours before linearly-increasing concentrations of linezolid were added (up to 10 mg/L in 0.5 mg/L increments). Cultures were incubated for a further 17 hours before being subcultured to fresh broth and left to grow before linezolid was added. After multiple passages, mutants able to grow in the presence of 10 mg/L linezolid were transferred to BHI agar containing 10 mg/L linezolid and were passaged five times at this concentration, in an attempt to stabilize resistance. Mutants were characterized by MIC determinations, and PFGE was performed to confirm parentage.

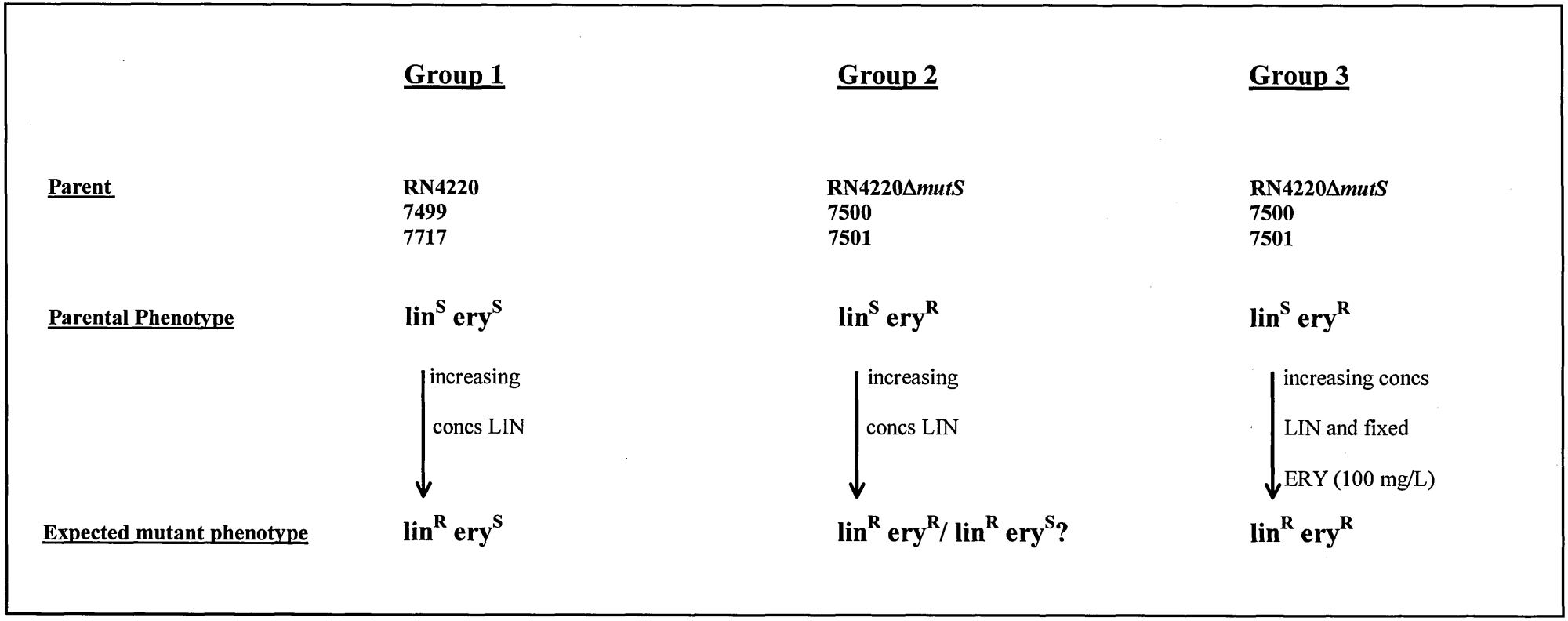
2.5.1.2 Method B

Strains were grown on BHI agar plates containing increasing concentrations of linezolid (in 2 mg/L increments, from 2 to 10 mg/L). Agar plates were incubated at 37°C for up to five days before colonies were picked off and passaged on agar containing the same or an increased concentration of linezolid. Once mutants were able to grow on BHI agar containing 10 mg/L linezolid, they were passaged five times at this concentration, in an attempt to stabilize resistance. Mutants were characterized by MIC determinations and PFGE to confirm parentage.

2.5.2 Generation of linezolid-resistant isolates in the presence of erythromycin

Erythromycin-resistant and -susceptible pairs of genetically-related isolates were grown on BHI agar containing increasing concentrations of linezolid (in 2 mg/L increments, up to 10 mg/L) in the presence or absence of erythromycin (100 mg/L) (see Figure 8 for details). Mutants were characterized by MIC determination, PFGE to confirm parentage, and PCR with primers *erm*(B)

Figure 8. Method used to generate linezolid (LIN)-resistant mutants in the presence of erythromycin (ERY).



and *erm*(A/C) to confirm the presence or absence of *erm* determinants (see section 2.7.2, Table 9 and Table 10 for details of PCR cycling conditions and primers, respectively).

2.5.2.1 Degradation of linezolid

To ensure that the slow emergence of colonies on agar containing linezolid was due to resistance and not the result of linezolid degradation, a set of linezolid MIC determination plates were poured (range 0.064 to 256 mg/L) and kept at 37°C for 5 days. On the fifth day, a second set of linezolid MIC determination plates were poured, both sets were dried and inoculated with strains with known linezolid MICs. Plates were incubated at 37°C for 24 hours. The two sets of MICs were read and compared.

2.5.3 Selection of chloramphenicol-resistant mutants

A similar methodology was employed to that used for raising linezolid-resistant mutants (see section 2.5.1.2). Strains were grown on BHI agar containing increasing concentrations of chloramphenicol (in 2 mg/L increments, from 2 to 16 mg/L). Once mutants could grow successfully at 16 mg/L chloramphenicol they were passaged five times at this concentration. Mutants were characterized by MIC determinations and PFGE.

2.6 Characterization of mutants of *S. aureus*

2.6.1 Extraction and purification of bacterial chromosomal DNA

BHI broth (5 ml) was inoculated with a 10 µl loop of freshly-grown bacteria and incubated overnight at 37°C with gentle shaking. Bacteria were harvested from 700 µl of this overnight growth and re-suspended in 100 µl salt/EDTA buffer (2.5 M NaCl, 50 mM EDTA, pH 7.5) to which was added 50 µl lysostaphin (stock solution of 1 mg/ml) and 100 µl lysozyme (stock solution of 100 mg/ml) and incubated at 37°C for a minimum of 1 hour. After lysing with 500 µl of GES (5.0 M guanidium thiocyanate, 0.1 M EDTA, 0.5% [w/v] sarkosyl), 250 µl of ammonium

acetate (7.5 M) was added, mixed and left on ice for 10 minutes. Phenol:chloroform:isoamyl alcohol (25:24:1, saturated with 10 mM Tris, pH 8.0, 1 mM EDTA), 500 µl, was then added and mixed well by hand for 10 minutes. The two phases were separated by centrifugation at 18300 x g for 10 minutes. The upper phase, 350 µl, was added to 875 µl of ice-cold ethanol and kept at -20°C for a minimum of one hour. DNA was spooled on to a 1 µl loop, washed with 1 ml ammonium acetate mix (1.5 M ammonium acetate in 70% ethanol) followed by 1 ml 100% ethanol, dried, and dissolved overnight at 4°C in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Bacterial chromosomal DNA preparations were stored at -20°C.

2.6.2 Long and accurate PCR

Long PCR was used to confirm the presence or absence of the pGhost9^{+} plasmid inserted to disrupt the *mutS* gene in RN4220Δ*mutS* and its descendants. A mastermix containing 1 µl (stock 1 µg/µl) forward primer, 1 µl (stock 1 µg/µl) reverse primer, 1.75 µl of each of dATP, dCTP, dGTP, dTTP, 5 µl of 10 x PCR buffer (containing 17.5 mM $MgCl_2$), 0.75 µl of Expand Long Template PCR mix (Roche) and 34.25 µl of PCR-quality water (Sigma) was prepared, vortexed and centrifuged briefly. A final reaction volume was achieved by adding 1 µl of chromosomal DNA preparation (see section 2.6.1) to 49 µl of mastermix. Tubes were vortexed and centrifuged briefly before being transferred to a thermocycler (P x 2 Thermocycler [Thermo]). Details of PCR cycling conditions can be seen in Table 9 and PCR primers in Table 10; see section 2.7.3 for analysis of PCR products.

2.6.3 Pulsed-field gel electrophoresis (PFGE)

S. aureus strains were grown overnight on NA at 37°C, then suspended in 1 ml of SE buffer (75 mM NaCl, 25 mM EDTA, pH 7.5) to a density equivalent to a McFarland suspension of 2.4 - 2.6 using a spectrophotometer (Densimat). Bacterial suspension, 400 µl, was mixed with 400 µl of 2% low-gelling agarose (dissolved in SE buffer) at 56°C and dispensed into moulds (five replicates per isolate). Moulds were kept at 4°C until set. Agarose plugs, from the moulds, were incubated in 3

ml gram-positive lysis buffer (6 mM Tris, 100 mM EDTA, 1M NaCl, 0.5% w/v Brij 58, 0.25 w/v sodium deoxycholate, 0.5% N-lauroyl sarcosine, 1 mM $MgCl_2$) containing 30 units/ml lysostaphin and 500 µg/ml lysozyme at 37°C, overnight, with gentle shaking. The lysis buffer was replaced with 3 ml gram-negative buffer (1% (w/v) N-lauroyl sarcosine, 0.5 M EDTA pH 9.5) containing 3.6 µl proteinase K (stock solution of 50 mg/ml) and incubated overnight at 56°C with gentle shaking. Agarose plugs were washed three times in 3 ml amounts of TE buffer. A portion of each plug was cut (approx 2 mm), covered with 100 µl reaction buffer (33 mM Tris acetate, 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM dithiothreitol) and incubated at 4°C for a minimum of 30 minutes. The buffer was then replaced with fresh reaction buffer to which was added 2 µl of *Sma*I restriction endonuclease (10 units/µl). Incubation was at 30°C for a minimum of 4 hours. A 1.2% agarose gel (molecular grade) was prepared in 0.5 x TBE buffer. Digested plugs were loaded into the wells along with molecular weight markers (lambda concatamer ladder) and the wells were sealed with molten agarose. The gel was placed into an electrophoresis tank (CHEF DR II apparatus) and 2 litres of electrophoresis buffer (0.5 TBE buffer), cooled to 4°C, were added. Standard running conditions were 6 V/cm, 12°C for 30 hours, switch times ramping from 1 to 80 seconds. Running conditions were altered to 6 V/cm, 12°C for 23 hours, with switch times ramping from 1 to 15 seconds in order to resolve fragments of 10-150 Kb. The PFGE gel was stained for 90 minutes with ethidium bromide (1 µg/ml), destained for 1 hour with distilled water, visualised under UV light, photographed and the image was stored electronically as .tif and .jpeg files.

2.7 Detecting and characterizing chromosomal mutations conferring antibiotic resistance

2.7.1 Primer design

Primers were designed manually using GenBank sequences (www.ncbi.nlm.nih.gov), accession numbers BA 00018 (*S. aureus* N315 complete genome) and X68425 (*S. aureus* gene for 23S rRNA). The genome sequence of NCTC 8325 (http://micro-gen.ouhsc.edu/s_aureus/s_aureus_

home.htm) was also used. Two primers were designed for each gene, spanning an area of interest. Forward and reverse primers were designed with two to three A or T bases at the 5' end and with two to three G or C bases at the 3' end. Primers were designed to be approximately 20 bp in length and to have a melting temperature of between 55°C and 65°C. Primers were checked for secondary structure, self-annealing and melting temperature using an on-line oligo calculator (www.basic.northwestern.edu/biotools/oligocalc.html). Primers were also checked for specificity using the on-line BLAST tool (Basic Local Alignment Search Tool) (www.ncbi.nlm.nih.gov/BLAST).

2.7.2 PCR amplification of 23S rRNA genes and detection of antibiotic resistance genes

S. aureus strains were grown overnight on NA at 37°C. Two colonies were suspended in 100 µl of PCR-quality water. Tubes were vortexed and centrifuged briefly. A PCR mastermix was prepared (78 µl PCR quality water, 10 µl 10 x *Taq* polymerase buffer, 5 µl Buffer W1, 4 µl $MgCl_2$ [50 mM], 2µl dNTP mix [2.5 µl of each of dATP, dGTP, dTTP, dCTP], 4 µl primer mix [5 µl of each primer; stock concentration of 1 µg/µl and 90 µl PCR quality water] and 0.5 µl *Taq* polymerase) for four reactions (or multiples of), vortexed and centrifuged briefly. A final reaction volume was achieved by adding 23 µl of the PCR mastermix to 2 µl of the template DNA. Tubes were vortexed and centrifuged briefly before being transferred to a thermocycler. PCR was also carried out in a 96-well PCR plate. Mastermix volumes were scaled-up appropriately and aliquoted into a plate using a multichannel pipette. Details of PCR cycling conditions and primers are given in Table 9 and Table 10 respectively.

2.7.3 Analysis of PCR products

Agarose gels, of suitable percentage (0.7-2.5%) were prepared by addition of 0.5 x TBE buffer to molecular grade agarose. Gel loading dye, 4 µl, (50 mM EDTA pH 8, 25% ficoll, 0.25% bromophenol blue) was added to 4 µl of the PCR product and loaded on to the gel. An appropriate

Table 9. PCR cycling conditions.

Program Name	Step I Denaturation period	Step II Denaturation, Primer annealing, Primer extension.	Step III Final extension	Reference
Techme 5	94°C for 5 min	30 cycles of: 94°C for 25 secs 52°C for 40 secs 72°C for 50 secs	72°C for 6 min	This work
erm(B)	94°C for 3 min	25 cycles of: 94°C for 60 secs 52°C for 60 secs 72°C for 60 secs	72°C for 10 min	(Soltani *et al.*, 2000)
erm(A/C)	94°C for 10 min	30 cycles of: 94°C for 30 secs 52°C for 30 secs 72°C for 60 secs	72°C for 10 min	(Lina *et al.*, 1999b)
Long PCR	94°C for 2 min	10 cycles of: 94°C for 10 sec 64°C for 30 sec 68°C for 8 min 20 cycles of: 94°C for 10 sec 64°C for 30 sec 68°C for 8 min	68°C for 7 min	(O'Neill and Chopra, 2002)
Sequencing	96°C for 3 min	30 cycles of: 96°C for 20 secs 50°C for 20 secs 60°C for 4 min	N/A	Joanna Craggs, Beckman Coulter (personal communication)

Table 10. PCR primers.

Forward Primer Reverse Primer	Forward Primer (5'-3') Reverse Primer (5'-3')	Target	Size of PCR amplicon	Program[A]	Reference
LRE 5 **LRE 6**	AACGATTTGGGCACTGTCTC TCCCGGTCCTCTCGTACTAA	23S rRNA genes (2005-2698)	694 bp	Techme 5	Neil Woodford (personal communication)
***erm* B F** ***erm* B R**	CATTTAACGACGAAACTGGC GGAACATCTGTGGTATGGCG	*erm*(B)	425 bp	Erm B	(Soltani *et al.*, 2000)
***erm* A/C F** ***erm* A R** ***erm* C R**	GTTCAAGAACAATCAATACAGAG GCTAATATTGTTTAAATCGTCAATTCC GGATCAGGAAAAGGACATTTTAC	*erm*(A/C)	*erm*(A) 421 bp *erm*(C) 572 bp	Erm A/C	(Lina *et al.*, 1999b)
MutS Int I **MutS Int II**	CGCATATCGAGGATGTTGTTCAATA GCTTTGTCTTCCGCACCTAAAAT	*mutS* gene	6.1 Kb	Long PCR	(O'Neill and Chopra, 2002)
23S rRNA VF **23S rRNA VR**	GCGGTCGCCTCCTAAAAG ATCCCGGTCCTCTCGTACTA	23S rRNA genes (2280-2699)	420 bp	Techme 5	(Pillai *et al.*, 2002)
F1 **R1**	ATTAAGTTATTAAGGGCGCACG TTACCTGAACTTCAACCTGACC	23S rRNA genes (2-762)	761	Techme 5	This work (see section 2.7.1)
F2 **R2**	TTTGTAGAATGAACCGGCG ATTCTCACTTCTAAGCGCTCC	23S rRNA genes (610-1293)	684	Techme 5	This work
F3 **R3**	AAACATATTACCGAAGCTGTGG TTACGGTTTAGCAGAGACC	23S rRNA genes (1186-1837)	652	Techme 5	This work

Cont.

Table 10. PCR primers (cont.).

Forward Primer Reverse Primer	Forward Primer (5'-3') Reverse Primer (5'-3')	Target	Size of PCR amplicon	Program[A]	Reference
F4 **R4**	AAAGCCTCTAGATAGAAAATAGG AGTCAAGCTCCCTTATGCC	23S rRNA genes (1617-2375)	759	Techme 5	This work
F5 **R5**	TTATCGTGGTGGGAGACAG TTGATTAAGTCTTCGATCGATTAG	23S rRNA genes (2237-2923)	687	Techme 5	This work

[A] See Table 9

linear size marker was also loaded (123 bp or 1 Kb ladder). Electrophoresis was carried out at 120-140V for approximately 80-110 minutes. Agarose gels were stained with ethidium bromide (1 µg/ml), visualised under UV light, photographed and the images stored electronically as .tif and .jpeg files.

2.7.4 Detection of linezolid resistance mutations by PCR-RFLP analysis

RFLP analysis of PCR amplicons was used to identify known mutations that confer resistance to linezolid. A 694 bp 23S rDNA PCR amplicon was generated (see section 2.7.2) using primers LRE 5 and LRE 6 (Table 9 and Table 10), and visualised as described previously (see section 2.7.3). PCR product, 8 µl, an appropriate restriction endonuclease (Roche or Fermentas), 1 µl, and an appropriate enzyme buffer, 1 µl, were vortexed and centrifuged briefly before being incubated at 37°C for a minimum of 4 hours. Restriction enzymes *Nhe*I (Woodford *et al.,* 2002), *Eco*RV (Lobritz *et al.,* 2003) and *Hin*1I (this work), were used to detect G2576T, G2505A and T2504C mutations, respectively, in the genes encoding 23S rRNA. Digested product, 8 µl, was analysed by gel electrophoresis (2.5% agarose gel), stained with ethidium bromide (1 µg/ml), visualised under UV light, photographed and stored electronically as .tif and .jpeg files.

Substitution of thymine for guanine at position 2576 (*E. coli* numbering) creates a new cutting site for *Nhe*I at 526 bp (on the 694 bp fragment). If no mutation is present, all copies of the *S. aureus* 23S rRNA genes are wild-type G and *Nhe*I will cut once producing fragments of 526 bp and 168 bp. If all copies are mutated T, *Nhe*I will cut twice producing fragments of 430 bp, 168 bp and 96 bp. If the mutant is heterozygous, with some copies wild-type and some mutated, digest with *Nhe*I provides a mixture of all of the fragments, 526 bp, 430 bp, 168 bp and 96 bp (see section 3.2.2, Figure 22).

The presence of a T2504C mutation results in the introduction of a new cutting site for *Hin*1I at 516 bp (on the 694 bp fragment) (See section 3.2.2, Figure 24). If no mutation is present, all copies of the 23S rRNA genes are wild-type T and *Hin*1I will not cut the 694 bp fragment. If all copies are mutated C, *Hin*1I will cut once producing fragments of 516 bp and 178 bp. If the mutant is

heterozygous, with some copies wild-type and some mutated, digest with *Hin*1I provides a mixture of all of the fragments, 694 bp, 516 bp, 178 bp.

Substitution of adenine for guanine at position 2505 (*E. coli* numbering) creates a new cutting site for *Eco*RI at 526 bp (on the 694 bp fragment). If no mutation is present, all copies of the 23S rRNA genes are wild-type G and *Eco*RI will not cut. If all copies are mutated A, *Eco*RI will cut once, producing fragments of 528 bp and 166 bp. If the mutant is heterozygous, with some copies wild-type and some mutated, digest with *Eco*RI provides a mixture of all of the fragments, 528 bp and 166 bp (see section 3.4.1, Figure 40).

2.7.5 Designing PCR-RFLP assays

A T to C change at position 2504 in the 23S rDNA genes was detected by DNA sequencing (see section 2.7.6). Both the wild-type sequence (T2504) and the mutant sequence (C2504) were loaded into a restriction analysis website (http://rna.lundberg.gu.se/cutter2/) and theoretically 'cut' with all enzymes represented in the database. The restriction sites of the two sequences were compared. One enzyme, *Hin*1I, would only cut the mutant sequence and could, therefore, be used to detect the presence of a T2504C mutation in the 23S rDNA genes. This method of detecting polymorphisms could only be used if a mutation introduced a new cutting site or caused loss of a site.

2.7.6 Partial DNA sequencing of 23S rRNA genes

DNA to be sequenced was first amplified with primers LRE 5 and LRE 6 (Table 9 and Table 10) to generate a 694 bp fragment of the genes encoding 23S rRNA (see section 2.7.2). PCR amplicons were used for gel electrophoresis, as previously described (see section 2.7.3). PCR products were purified using a Q-BIOgene Geneclean Turbo Kit (Q-BIOgene). Purified DNA (1-2 µl) and PCR quality water (to a final volume of 10 µl) were vortexed in a 96-well sequencing plate, centrifuged and denatured in a thermal cycler at 96°C for 3 minutes. A relevant primer, forward or reverse (0.64 µl [stock concentration of 1 µg/µl]) was added, along with Dye Terminator Cycle Sequencing (DTCS) quick start mix (8 µl), giving a total reaction volume of 20 µl. This mix was vortexed and centrifuged briefly before being returned to the thermal cycler. After amplification, 5

µl of stop mix (0.4 µl 0.5M EDTA, 1.6 µl PCR water, 2.0 µl 3M sodium acetate, 1.0 µl glycogen) was added to each well, along with 60 µl of 95% ice-cold ethanol. The plate was vortexed and centrifuged at 1110 x g for 30 minutes at 4°C, then inverted to remove the ethanol. Ice-cold ethanol, 200 µl (70%) was added to each well and centrifuged at 1110 x g for 10 minutes at 4°C. The plate was inverted to remove the ethanol and the step repeated. The plate was again inverted to remove the ethanol and spun inverted at 10 x g for 15 seconds. The plate was removed, turned upright and was left for a minimum of 45 mins to dry at room temperature. Sample loading solution, 40 µl, was added to each well, vortexed well and centrifuged. One drop of mineral oil, from a pasteur pipette, was added to each well and the plate was loaded into the sequencing machine (Beckman CEQ8000 automated sequencer).

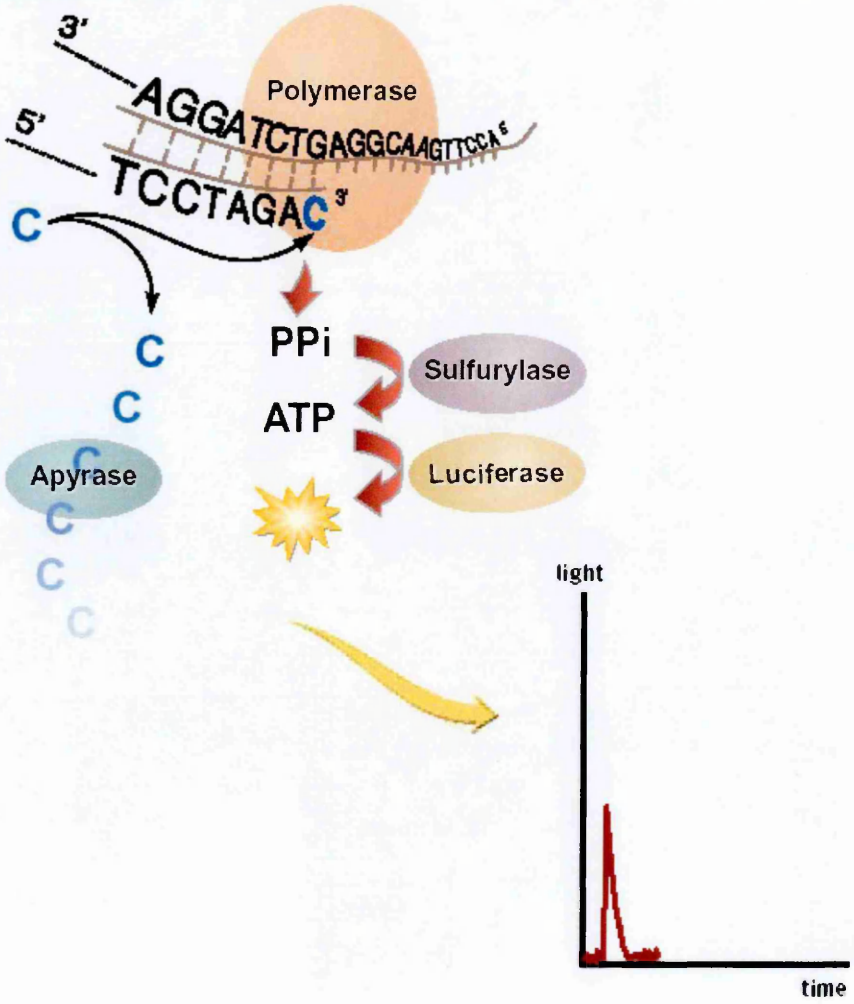
2.7.6.1 Analysis of chromatograms

Sequencing results were imported into Bioedit and Bionumerics software programs (www.applied-maths.com). These programs were used to align multiple sequence reads to produce a consensus sequence. The on-line BLAST tool was used for comparing sequence results with GenBank sequences.

2.7.7 Pyrosequencing

Pyrosequencing is a technique whereby a PCR product is generated, spanning the region of interest, using a forward primer, a reverse primer with a 21 bp handle attached, and a universal biotinylated primer, with a sequence matching that of the handle. The biotinylated primer is incorporated into one strand of the PCR product. A clean-up mechanism uses streptavidin beads to capture the biotinylated strand. A sequencing primer binds to the captured template strand. Nucleotides are dispensed in an order specified by the user, according to known sequence and mutations to be detected. Successful incorporation of a nucleotide, complementary to the template strand, initiates a cascade of events resulting in the emission and detection of light (see Figure 9). A peak is produced on the pyrogram® that is proportional to the amount of nucleotide incorporated.

Figure 9. Pyrosequencing cascade. A sequence primer hybridizes to a single-stranded PCR product. The first dNTP is added. DNA polymerase catalyses the incorporation of the dNTP if it is complementary to the template. Each successful incorporation of a nucleotide is accompanied by the release of pyrophosphate (PPi). A cascade reaction occurs which results in the emission of light, which is subsequently detected. The amount of light emitted is proportional to the number of nucleotides incorporated. Unincorporated nucleotides are degraded before the next nucleotide is added (From http://www.pyrosequencing.com/graphics/3019.pdf).



2.7.7.1 Pyrosequencing assay design

A pyrosequencing assay was designed to detect the presence of seven known mutations conferring resistance to linezolid. A 364 bp pair region of the 23S rDNA genes (2411 to 2775) was amplified with three primers (see Figure 10);

- a normal forward primer 5'AAGGGCCATCGCTCAACGGAT
- a reverse primer, with a universal handle sequence (Mostafa Ronaghi [Stanford University], personal communication) added to the 5' end
  5'(CCGAATAGGAACGTTGAGCCT)GTGAGGGGGGCTTCATGCTT
- a universal biotinylated primer, with a sequence matching that of the handle
  5'-biotin-CCGAATAGGAACGTTGAGCCT

Initially, pyrosequencing software was used to identify possible primer regions, but subsequently primers were designed manually (see section 2.7.1). A reverse primer was designed, and the 5' handle added. The handle was an exact match of the universal biotinylated primer. This resulted in the integration of biotin into the reverse strand of the PCR product, which was then captured during the post-PCR clean-up. A forward sequencing primer was designed to anneal to the captured reverse template strand. The resulting pyrogram could then be read in the forward direction. Three sequencing primers were designed, spanning the seven mutations of interest (see Table 11). Sequencing primers were designed to anneal within five base pairs of the first mutation to be detected. All sequencing primers were between 17 and 20 base pairs in length and had a melting temperature of 57-58°C.

2.7.7.2 Pyrosequencing assay

A PCR reaction master mix was set up, as before (see section 2.7.2), but with forward, reverse and biotinylated primers (5 µl of forward and reverse primers [stock concentration 1 µg/µl], 10 µl universal biotinylated primer [stock concentration 1 µg/µl] and 80 µl PCR quality water). The mastermix (23 µl) was multi-channelled into a PCR plate. A final reaction volume was achieved by adding 2 µl of the template DNA. PCR products were visualised as before (see section 2.7.3).

Figure 10. 364 bp amplified region of 23S rRNA. PCR primers are underlined, sequencing primers are highlighted in blue, positions of mutations are highlighted in red.

AAGGGCCATCGCTCAACGGATAAAAGCTACCCCGGGGATAACAGGC

TTATCTCCCCCAAGAGTTCACATCGACGGGGAGGTTTGGCACCTCGA

TGTCGGCTCATCGCATCCTGGGGCTGTAGTCGGTCCCAAGGGTTGGG

CTGTTCGCCCATTAAAGCGGTACGCGAGCTGGGTTCAGAACGTCGTG

AGACAGTTCGGTCCCTATCCGTCGTGGGCGTAGGAAATTTGAGAGGA

GCTGTCCTTAGTACGAGAGGACCGGGATGGACATACCTCTGGTGTAC

CAGTTGTCGTGCCAACGGCATAGCTGGGTAGCTATGTGTGGACGGGA

TAAGTGCTGAAAGCATCTAAGCATGAAGCCCCCCTCAC

Table 11. Pyrosequencing sequencing primers.

Sequence to be detected	Mutation detected	Primer 5'-3'
CTCGATGTCGG	T2500A, A2503G, T2504C, G2505A	ACGGGGAGGTTTGGCAC
GGGATAACA	G2445T, G2447T	AACGGATAAAAGCTACCCCG
GCGAGC	G2576T	TTCGCCCATTAAAGCGGTAC

Three repeat assays were performed for each isolate, with each of the sequencing primers. Binding buffer (40 µl per sample) (10 mM tris-HCl, 2M NaCl, 1mM EDTA, 0.1% tween 20), streptavidin sepharose bead mix (2 µl per sample) and PCR quality water (20 µl per sample) were added to 20 µl of PCR product and agitated for 10 minutes at room temperature (1400 rpm/3 mm mixing stroke). A 96-well pyrosequencing plate containing annealing buffer, 44.2 µl per sample, (20 mM Tris, 2 mM magnesium acetate, pH 7.6) and sequencing primer (0.8 µl per sample [0.14 nmols added]) was prepared. Strand separation was achieved using a vacuum preparation tool. Probes of the vacuum tool were washed with high purity water (20 seconds), before being moved to the PCR plate. Beads containing immobilized template were captured on the probes. The vacuum preparation tool was moved to a trough containing 70% ethanol and flushed through for 5 seconds. Next, denaturation solution (0.2M NaOH) was flushed through the probes for 5 seconds; finally, washing buffer (10 mM tris-acetate, pH 7.6) was flushed through the probes for 5 seconds. The vacuum was closed and the beads were released into the plate containing annealing buffer and sequencing primer master mix. The vacuum tool was washed through for 30 seconds with high purity water. The plate, now containing the beads, annealing buffer and sequencing primer, was heated at 80°C for 2 minutes before cooling slowly to room temperature, then the plate was loaded into the pyrosequencing machine. Freeze-dried enzyme (DNA polymerase, ATP sulfurylase, luciferase and apyrase) and substrate (adenosine 5' phosphate and luciferin) mixtures were reconstituted using 620 µl of PCR quality water and left at room temperature for 5-10 minutes to dissolve. The amounts of enzyme or substrate required were calculated (50 µl + [5.5 x no. of wells]) and loaded into the reagent cartridge. The amount of nucleotides required (dCTP, dGTP, dTTP and dATPαS) was calculated (50 µl + [0.11 x 7 x no. of wells]), diluted with an equal amount of TE buffer and loaded into the reagent cartridge. The cartridge was loaded into the pyrosequencing machine and the run started. Controls were run alongside samples to be tested. Sequencing primer (no DNA added) was used to detect background signal. Negative PCR product was used to check a background signal was not being generated from the biotin-labelled PCR product. In one well a PCR fragment (no sequencing primer was added) was run to detect the presence of any hairpin loops within the PCR product. Additionally water was substituted in place

of a PCR product to check for contamination. The results of the pyrosequencing run were analysed using pyrosequencing software to detect and quantify the presence of mutations.

2.8 DNA hybridization

2.8.1 Digestion of chromosomal DNA for hybridization

DNA was extracted and purified as described in section 2.6.1. Chromosomal DNA preparation, 6-8 µl *Eco*RI, 1 µl enzyme buffer (50 mM Tris-HCL, 10 mM magnesium chloride, 100 mM sodium chloride, 1 mM dithiothreitol, pH 7.5), 1 µl, and RNase, 1 µl (1 mg/ml), were vortexed, centrifuged briefly and incubated at 37°C overnight. Digestion products were analysed by gel electrophoresis (0.8% [w/v] agarose gel), stained with ethidium bromide (1 µg/ml), visualised under UV light, photographed and stored electronically as .tif and .jpeg files.

2.8.2 Vacuum blotting

A VacuGene blotting unit was assembled. The porous support was pre-wetted with distilled water. Hybond-N nylon membrane was pre-cut to the appropriate size, pre-wetted by immersing in distilled water and placed on the porous support under a plastic mask. The gel (see section 2.8.1) was placed on to the membrane and the vacuum applied at 45-50 mBar. Enough fragmenting solution (0.25 M HCl) to cover the gel was applied for eight minutes. The excess was removed before denaturing solution (0.5 M NaOH, 1.5 M NaCl) was added for six minutes, followed by neutralizing solution (1.5M NaCl, 0.5 M Tris, 1 mM EDTA, pH 7.2) for six minutes. Next, 20 x salt sodium citrate (SSC) transfer solution (175.3g NaCl, 88.2g sodium citrate, water to 1 litre, pH 7.0) was added for 60-90 minutes. A UV cross-linker was used to covalently bond DNA to the membrane, which was then left to dry at room temperature. The membrane and gel were viewed under UV light to ensure the DNA had transferred. The membrane was stored at room temperature.

2.8.3 Preparation of digoxigenin-labelled probes by PCR

A primary PCR reaction (25 µl volume) (see section 2.7.2) amplified a 420 bp region of 23S rDNA genes (primers 23S rRNA V F and R) (see Table 9 for PCR conditions and Table 10 for PCR primers). The resulting PCR products were analysed by gel electrophoresis, stained with ethidium bromide (1 µg/ml) and visualised under UV light. The primary PCR product was used in a secondary PCR reaction. The secondary PCR reaction was scaled up to a 100 µl volume, the volume of dTTP was reduced and digoxigenin-11-dUTP incorporated. The ratio of unsubstituted dTTP to labelled dUTP was 2:1. A PCR mastermix was prepared, vortexed and centrifuged (62.5 µl PCR quality water, 10 µl 10 x *Taq* polymerase buffer, 5 µl Buffer W1, 4 µl $MgCl_2$ [50 mM], dNTP mix [2.5 µl dATP, 2.5 µl dGTP, 2.5 µl dCTP and 1. 7 µl dTTP], 8.3 µl dig-11-dUTP), primer mix 1 µl [stock 1 µg/µl] of each of the two primers], 1 µl of the primary PCR product and 1 µl *Taq* polymerase). PCR products were analysed by gel electrophoresis alongside the product from the primary PCR reaction, then stained with ethidium bromide (1 µg/ml), visualised under UV light, photographed and stored electronically as .tif and .jpeg files. Successful incorporation of digoxigenin slowed migration through the agarose gel, and the secondary PCR product appeared 'larger' than the primary product.

2.8.4 Hybridization studies

The membrane (see section 2.8.2) was placed in a hybridization bottle, immersed in 25 ml of pre-hybridization solution (16.25 ml water, 6.25 ml SSC [x 20], 50 µl SDS [10%], 83 µl sarkosyl [30%], 2.5 ml buffer set 3 [x 10] [Roche Applied Science, Lewes, UK]) and placed in a hybridization oven, pre-heated to 70°C, for a minimum of 1 hour. The pre-hybridization solution was replaced with 5 ml of fresh pre-hybridization solution to which had been added 30 µl of labelled probe DNA (see section 2.8.3) which had been denatured at 95°C for 2-3 minutes. The membrane was hybridized overnight at 70°C. After use, the probe mixture was stored at -20°C for future use and was re-used up to five times before being discarded. The hybridized membrane underwent two low stringency washes in the hybridization bottle with 50 ml 2 x SSC/0.1% SDS

(100 ml 20 x SSC, 10 ml 10% SDS [10g SDS, 100 ml water], 890 ml water) at room temperature for 5 minutes. The membrane was then washed twice with a high stringency solution, comprising 50 ml 0.1% SSC/ 0.1% SDS (5 ml 20 x SSC, 10 ml 10% SDS, 9985 ml water) at 70°C for 15 minutes. The remaining washes were all carried at room temperature. The membrane was rinsed with 100 ml washing buffer (100 ml buffer set I (x10), 900 ml distilled water). Next, the membrane was blocked for 30 minutes with 100 ml of blocking buffer (10 ml buffer set 2 [x10], 10 ml buffer set 3 [x10], 80 ml distilled water), before being rinsed for 1 minute in 100 ml washing buffer. Following this, the membrane was soaked for 30 minutes in anti-digoxigenin antibody conjugate, diluted 1:5000 in washing buffer, before being washed twice in washing buffer for 15 minutes. Next, the membrane was rinsed for 2-3 minutes in developing buffer (3.33 ml buffer set 4 [x10], 30 ml distilled water). Colour development reagent was prepared (400 µl NBT [nitro blue tetrazolium chloride] /BCIP [5-bromo-4-chloro-3-indolyl phosphate] in 10 ml developing buffer). The membrane was transferred to a hybridization bag and the colour development reagent was added, the bag sealed and stored in the dark. When bands had appeared to a suitable intensity, the membrane was washed in 200 ml of TE buffer (50 mM, pH 8.2) and dried at 70°C for 1 hour. Membranes were photographed and images stored electronically as .tif and .jpeg files.

2.9 Detection of hypermutators among clinical *S. aureus* isolates

2.9.1 Mutation frequencies

ISO broth, 10 mls, was inoculated using freshly-grown bacteria, and incubated overnight at 37°C. One ml of this overnight culture was used to inoculate 10 mls of fresh ISO broth, which was incubated with gentle shaking at 37°C for 5 hours. Viable counts (cfu/ml) were then determined. Serial dilutions from 10^{-1} to 10^{-8} were prepared in PBS, and 2 x 20 µl drops of 10^{-4} to 10^{-8} dilutions were plated on CBA and incubated at 37°C for 24 hours. The colony count was calculated as the arithmetic mean number of colonies present in 2 x 20 µl drops of the highest serial dilution with growth. This figure was multiplied by 50 to provide the number of cfu in 1 ml.

Mutants were selected by spreading 200 µl of the undiluted growth on to BHI agar, containing an appropriate concentration of antibiotic, and incubated at 37°C for 24 hours. The mutation frequency was calculated by obtaining an arithmetic mean number of colonies from four replicate antibiotic plates, multiplying by five (to obtain the number of mutants present in 1 ml) and dividing by the colony count. Experiments were carried out in triplicate. RN4220 was used as a wild-type control strain and RN4220Δ*mutS* as a hypermutable control strain.

2.9.2 Fosfomycin disc tests

Freshly-grown bacteria were suspended to a density of a 1.0 McFarland standard and swabbed over an ISO agar plate, containing 1 mg/ml G-6-P, to give confluent growth. A fosfomycin disc (50 µg) was placed on to the agar and incubated at 37°C for 24 hours. Three replicates were performed for each isolate. The numbers of colonies in the zone of inhibition around the fosfomycin disc were counted and the zone diameters measured (Galan *et al.*, 2003). RN4220 and RN4220Δ*mutS* were used as wild-type and hypermutable control strains, respectively.

3 Results

3.1 Characterization of reference strains

3.1.1 Hypermutable and wild-type reference strains

RN4220, a derivative of the laboratory strain NCTC 8325, was used as a parent strain during the creation of a hypermutable *mutS* deletion mutant (Giachino *et al.*, 2001). This hypermutable mutant, RN4220Δ*mutS*, was generated through the integration, via homologous recombination, of the plasmid, pGhost9$^+$, (containing some *mutS* sequence), creating a strain with an inactivated *mutS* gene and consequently a hypermutable phenotype (O'Neill and Chopra, 2002). The PFGE profiles of RN4220 and RN4220Δ*mutS*, after digestion with the restriction endonuclease *Sma*I, differed by one band (see section 2.6.3) (Figure 11). This difference between the two strains was characterized further.

To do this, contigs of the genome of NCTC 8325 were downloaded from http://micro-gen.ouhsc.edu/s_aureus/s_aureus_home_b.htm and, using BioEdit, were searched for the *mutS* and *mutL* genes. Once the genes were located, the appropriate contig was searched for *Sma*I sites. It was deduced that both genes were present on the largest fragment in the *Sma*I PFGE profile of RN4220, fragment A, approximately 674 Kb in size (Figure 12). The pGhost9$^+$ plasmid in RN4220Δ*mutS*, inserted to disrupt the *mutS* gene, itself has a *Sma*I cutting site present. Digest of the plasmid with *Sma*I linearises the plasmid, producing a fragment of 4.6 Kb (Figure 13) (Maguin *et al.*, 1996). This *Sma*I site within the plasmid resulted in the 674 Kb fragment A being split into two fragments of approximately ~520 Kb and ~155 Kb in RN4220Δ*mutS* (Figure 14). By changing the PFGE running conditions it was possible to resolve the smaller fragments and a ~150 Kb fragment could be identified in the hypermutable strain that is not present in the wild-type strain (Figure 15). This difference in PFGE profiles between the two strains proved a valuable tool for differentiating between the two strains and confirming parentage of mutants.

MICs for the two strains revealed that RN4220Δ*mutS* was resistant to erythromycin, due to the erythromycin-resistance marker present on the pGhost9$^+$ plasmid (see section 2.3.2) (Horinouchi and Weisblum, 1982). PCR confirmed the presence of an *erm*(B) determinant (Table 12) (see section 2.7.2). RN4220 was erythromycin-susceptible.

Figure 11. PFGE gel showing *Sma*I digested DNA from RN4220 and RN4220Δ*mutS* illustrating the differences in their banding patterns. Lane 1 48.5 Kb lambda ladder, lane 2 RN4220, lane 3 RN4220Δ*mutS*.

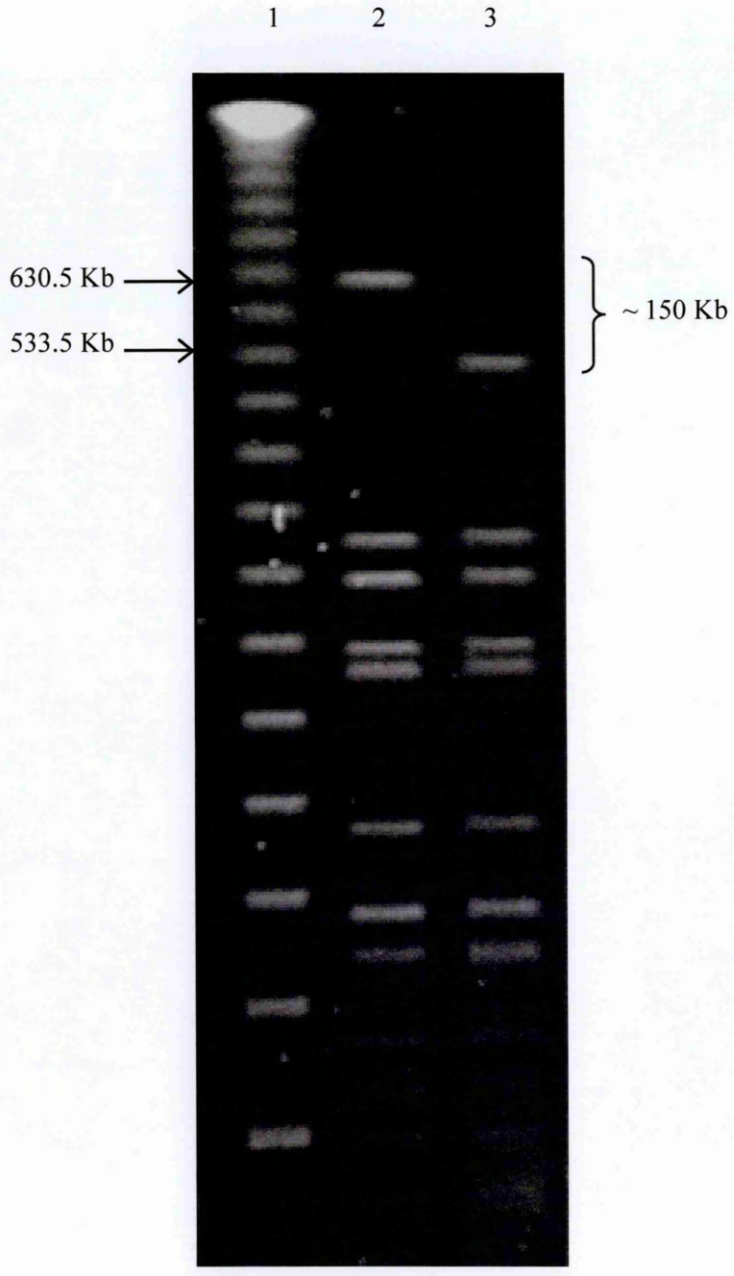


Figure 12. Restriction digest map of *S. aureus* NCTC 8325. The *mutS* and *mutL* genes are situated on fragment A (highlighted in blue). (Adapted from http://microgen.ouhsc.edu/s_aureus/ s_aureus _home_b.htm).

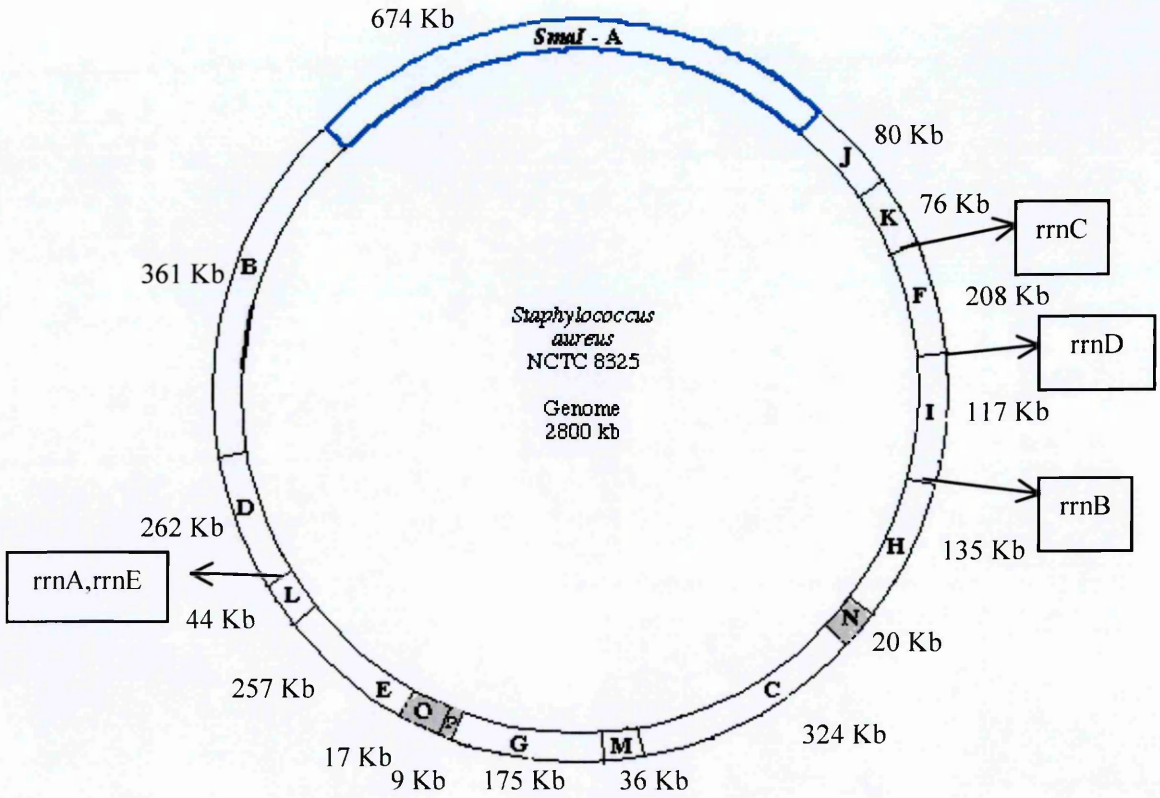


Figure 13. *Sma*I digest of pGhost9^{+}. Lane 1 1 Kb ladder, lane 2 undigested pGhost9^{+}, lane 3 pGhost9^{+} digested with *Sma*I, lane 4 water control.

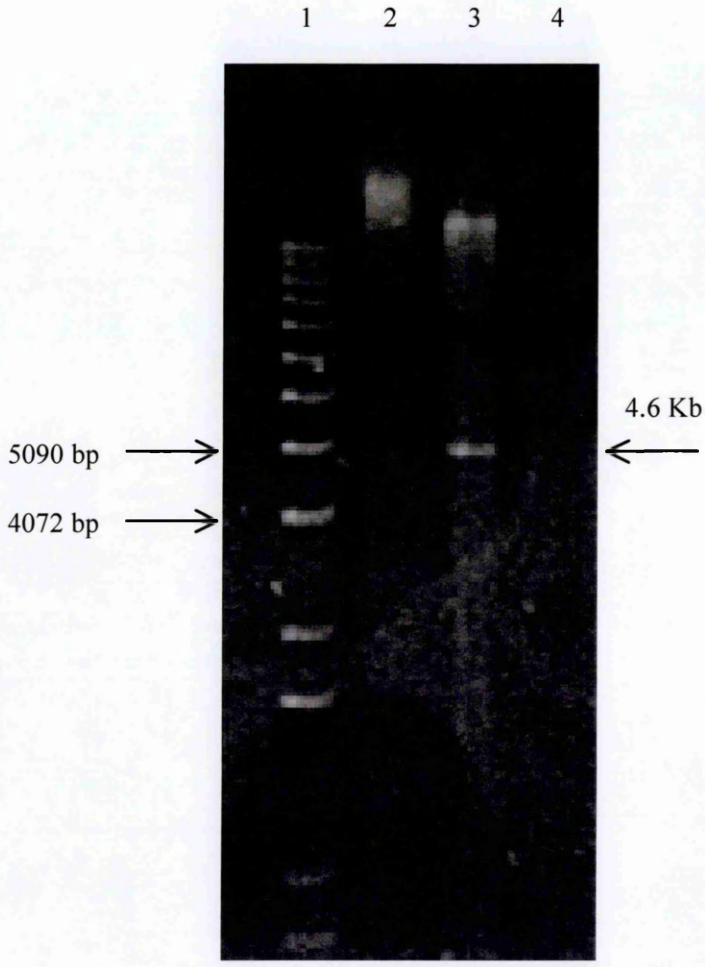


Figure 14. ***Sma*I cutting sites on fragment A of the PFGE profile of RN4220 and RN4220Δ*mutS*** **(not to scale).**

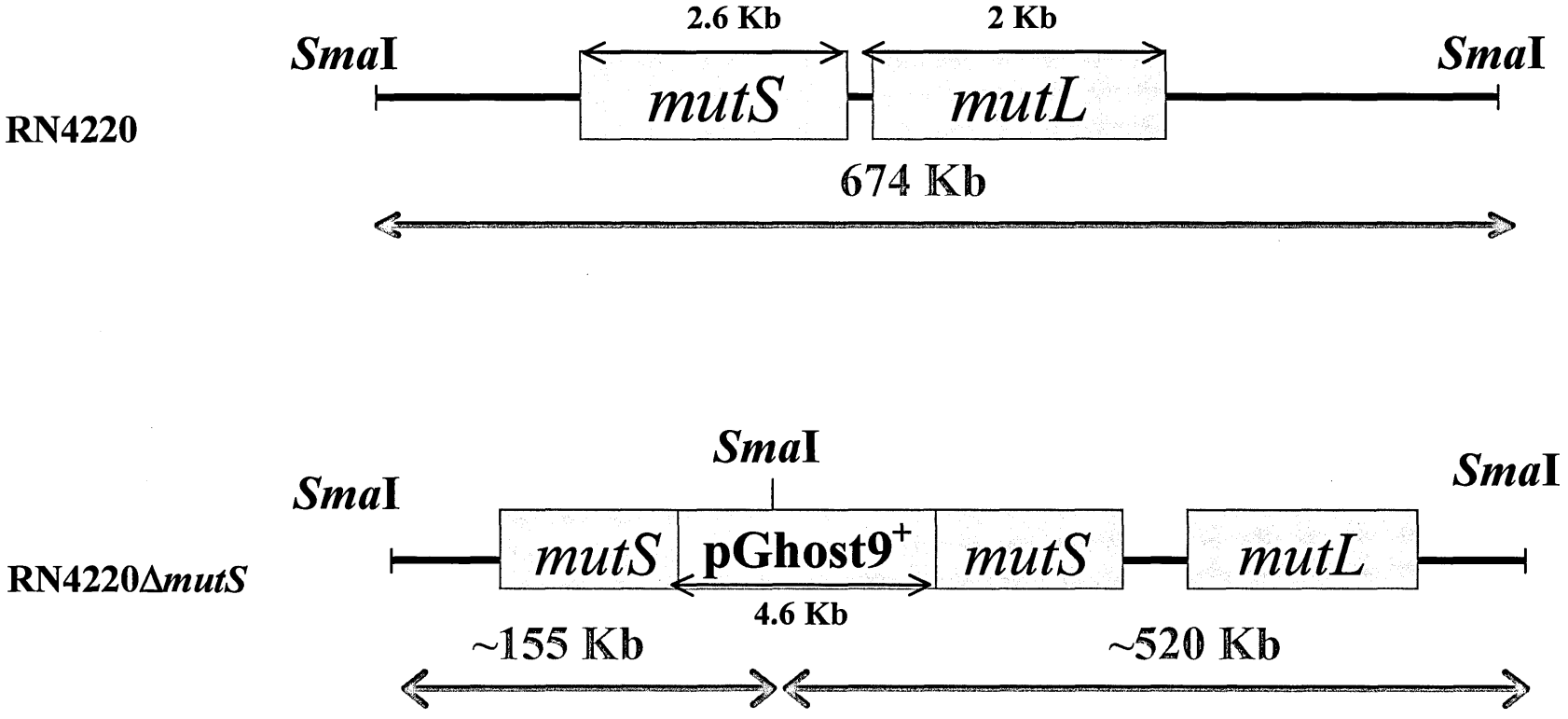


Figure 15. PFGE profiles of RN4220 and RN4220Δ*mutS* run with altered conditions to resolve smaller fragments. Lanes 1 and 4 RN4220Δ*mutS*, lanes 2, 3 and 5 RN4220, lane 6 48.5 Kb lambda ladder.

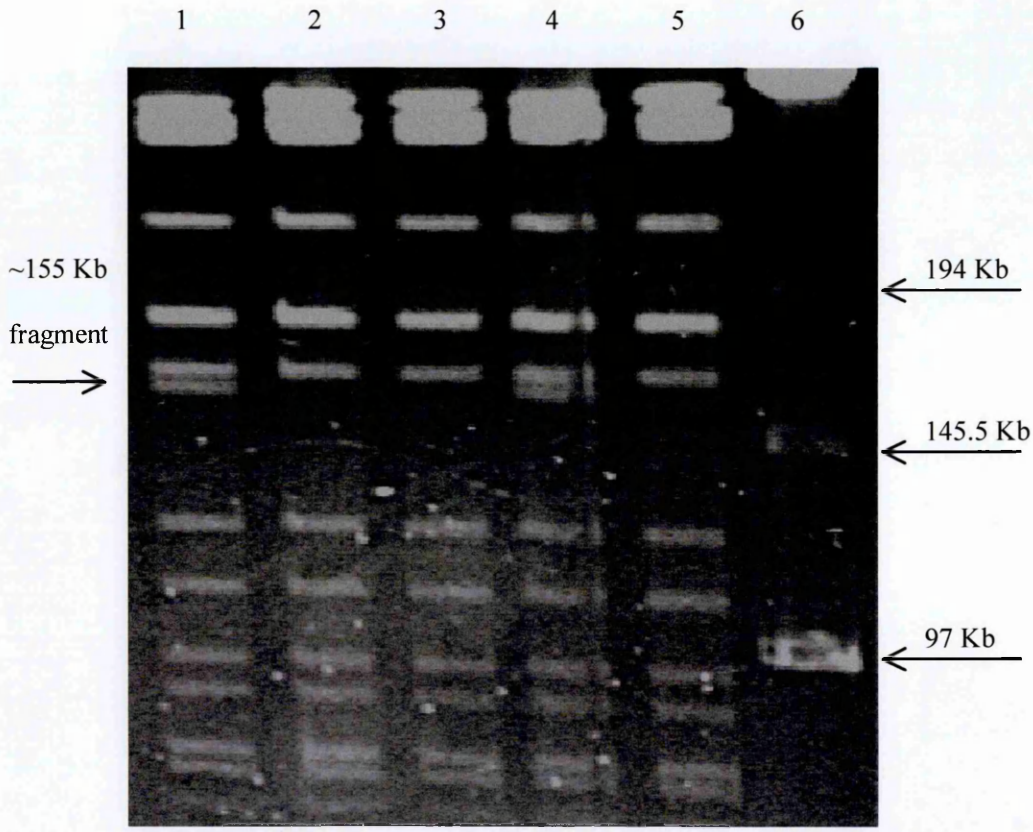


Table 12. MICs (mg/L) for wild-type, hypermutable and clinical strains of *S. aureus*.

Strain	Chlor[A]	Clin[B]	Ery[C]	Fus[D]	Lin[E]	Oxa[F]	Rif[G]	Teic[H]	Vanc[I]
RN4220	8	≤ 0.25	1	0.125	4	1	0.0016	1	2
RN4220Δ*mutS*	8	≤ 0.25	>256	0.064	4	0.5	0.0016	2	2
ST/03/2121	4	≤ 0.25	>256	0.125	4	>16	0.016	1	1
ST/03/2122	4	≤ 0.25	>256	0.064	1	>16	>256	8	4

[A]Chloramphenicol, [B]Clindamycin, [C]Erythromycin, [D]Fusidic acid, [E]Linezolid, [F]Oxacillin, [G]Rifampicin, [H]Teicoplanin, [I]Vancomycin.

3.1.2 Clinical reference strains

Two genetically-related clinical strains, isolated during therapy with teicoplanin - one teicoplanin-susceptible (ST/03/2121), MIC 1 mg/L, and the other teicoplanin-resistant (ST/03/2122), MIC 8 mg/L, - were identified as EMRSA-15 by phage typing and PFGE. MICs were determined by agar dilution (Table 12).

3.1.3 Mutation frequencies

Initially, the frequencies at which RN4220, RN4220Δ*mutS*, ST/03/2121 and ST/03/2122 selected mutants resistant to rifampicin (50 mg/L), fusidic acid (0.5 mg/L), teicoplanin (6 mg/L), vancomycin (6 mg/L) and linezolid (6 mg/L) were calculated (see section 2.9.1). Further mutation frequency experiments were carried out with the addition of sub-inhibitory concentrations of ciprofloxacin, teicoplanin, vancomycin and linezolid to the broths, both singularly and in combination. Mean mutation frequencies were calculated from a minimum of three replicated experiments.

Mutation frequencies revealed the hypermutable strain, RN4220Δ*mutS*, to mutate on average at the greatest frequency to all of the antibiotics and all but one of the antibiotic combinations tested (rifampicin and ciprofloxacin) at rates of 10^{-7} or 10^{-6} (Table 13). The two wild-type strains, RN4220 and ST/03/2121 mutated on average, at frequencies of 10^{-9} to 10^{-7}. The only measurable mutation frequency for the clinical isolate, ST/03/2122, was to fusidic acid. Intermediate or full resistance to the other antibiotics tested prevented the determination of mutation frequencies.

The hypermutable strain, RN4220Δ*mutS*, mutated to rifampicin at an average frequency of 5.4 x 10^{-7}, 11-fold greater than its wild-type parent, RN4220 (5.1 x 10^{-8}), and nine-fold greater than the clinical isolate, ST/03/2121 (5.9 x 10^{-8}) (Table 13 and in Appendix B, Table 44). The addition of a sub-inhibitory concentration of ciprofloxacin caused a two-fold increase in the mutation frequency of RN4220Δ*mutS* and a negligible difference in the mutation frequency of ST/03/2121, when compared with mutation frequencies to rifampicin (Appendix B Table 45). However, with the addition of ciprofloxacin, the mutation frequency of RN4220 jumped to 1.25 x 10^{-6}, a 25-fold

Table 13. Summary of mutation frequencies for RN4220, RN4220Δ*mutS*, ST/03/2121 and ST/03/2122. (See Appendix B, Table 44, Table 45, Table 46, Table 47, Table 48, Table 49 for more detailed results)

Antibiotic exposure (For MICs see Table 12)	**Mean mutation frequency and standard deviation**			
	RN4220	**RN4220Δ*mutS***	**ST/03/2121**	**ST/03/2122[B]**
Rif (50 mg/L)	$5.07 \pm 1.65 \times 10^{-8}$	$5.35 \pm 1.24 \times 10^{-7}$	$5.87 \pm 1.83 \times 10^{-8}$	N/A
Rif (50 mg/L) plus 0.25 mg/L cip in broth.	$1.25 \pm 1.25 \times 10^{-6}$ ↑ 25-fold	$8.50 \pm 3.75 \times 10^{-7}$ ↑ 2-fold	5.36×10^{-8} [A] ↓ 1-fold	N/A
Rif (50 mg/L) plus 0.25 mg/L cip, 0.5 mg/L teic in broth.	$6.23 \pm 3.29 \times 10^{-8}$ ↑ 1-fold	$7.45 \pm 2.19 \times 10^{-7}$ ↑ 1-fold	7.50×10^{-9} [A] ↓ 8-fold	N/A
Rif (50 mg/L) plus 0.25 mg/L cip, 0.5 mg/L vanc in broth.	$5.23 \pm 4.77 \times 10^{-7}$ ↑ 10-fold	$1.54 \pm 0.41 \times 10^{-6}$ ↑ 3-fold	1.54×10^{-8} [A] ↓ 4-fold	N/A
Rif (50 mg/L) plus 0.25 mg/L cip, 0.5 mg/L lin in broth.	$8.40 \pm 3.59 \times 10^{-8}$ ↑ 2-fold	$4.74 \pm 1.20 \times 10^{-7}$ ↓ 1-fold	1.43×10^{-7} [A] ↑ 2-fold	N/A
Fus (0.5 mg/L)	$3.48 \pm 1.48 \times 10^{-7}$	$3.52 \pm 0.95 \times 10^{-6}$	$4.95 \pm 0.83 \times 10^{-7}$	$5.74 \pm 3.52 \times 10^{-8}$
Teic (6 mg/L)	$7.63 \pm 3.38 \times 10^{-8}$	$1.52 \pm 1.20 \times 10^{-6}$	$7.03 \pm 1.39 \times 10^{-8}$	N/A
Vanc (6 mg/L)	$6.39 \pm 2.17 \times 10^{-9}$	$1.42 \pm 1.27 \times 10^{-6}$	2.54×10^{-9}	N/A

Cont.

Table 13. Summary of mutation frequencies for RN4220, RN4220Δ*mutS*, ST/03/2121 and ST/03/2122 (cont.)

Antibiotic exposure (For MICs see Table 12)	**Mean mutation frequency and standard deviation**			
	RN4220	**RN4220Δ*mutS***	**ST/03/2121**	**ST/03/2122**[B]
Lin (6 mg/L)[C]	$<6.66 \times 10^{-9}$ [C]	$<1.51 \times 10^{-8}$ [C]	$<1.92 \times 10^{-9}$ [C]	$<3.75 \times 10^{-9}$ [C]
Lin (6 mg/L) plus 0.5 mg/L in broth	$<1.34 \times 10^{-8}$ [C]	$<1.74 \times 10^{-8}$ [C]	$<5.56 \times 10^{-9}$ [C]	$<3.33 \times 10^{-9}$ [C]

[A] No average, only one data set.

[B] ST/03/2122 resistant to rifampicin, teicoplanin. Mutation frequencies to vancomycin were not measurable.

[C] Mutation frequency below average detectable limit.

increase on the mutation frequency to rifampicin. Closer analysis revealed one individual experiment with an undetectable mutation frequency (<2.17 x 10^{-7}), another with a frequency of 2.50 x 10^{-9} and a third anomalous mutation frequency of 2.50 x 10^{-6} (Appendix B, Table 45). The mutation frequencies for ST/03/2121 with the addition of sub-inhibitory concentrations of antibiotics were estimated only once.

The addition of sub-inhibitory concentrations of ciprofloxacin and teicoplanin, in combination, caused negligible differences in the mutation frequencies of RN4220Δ*mutS* (7.5 x 10^{-7}) and RN4220 (6.2 x 10^{-8}) to rifampicin. An eight-fold drop in the mutation frequency for ST/03/2121 was seen when compared with that seen for rifampicin singularly. The addition of sub-inhibitory concentrations of ciprofloxacin and vancomycin caused a three-fold increase in the mutation frequency of RN4220Δ*mutS* (1.5 x 10^{-6}), a ten-fold increase in that of RN4220 (5.2 x 10^{-7}) and a four-fold decrease in that of ST/03/2121 (1.5 x 10^{-8}) to rifampicin when compared with those seen for rifampicin alone. The addition of sub-inhibitory concentrations of ciprofloxacin and linezolid caused a two-fold increase in the mutation frequencies of RN4220 (8.4 x 10^{-8}) and ST/03/2121 (1.4 x 10^{-7}) and an insignificant shift in those of RN4220Δ*mutS* (4.7 x 10^{-7}), when compared with those seen for rifampicin. Overall, no consistent increases or decreases in the mutation frequency to rifampicin were seen with the addition of sub-inhibitory concentrations of ciprofloxacin and teicoplanin, vancomycin or linezolid.

The highest, average mutation frequencies to fusidic acid were seen for RN4220Δ*mutS* (3.5 x 10^{-6}) being ten- and seven-fold higher, respectively, than those seen for RN4220 (3.5 x 10^{-7}) and ST/03/2121 (5.0 x 10^{-7}) (Appendix B, Table 46). The average mutation frequency seen for RN4220Δ*mutS* to fusidic acid was 61-fold higher than that for ST/03/2122 (5.7 x 10^{-8}). Mutation frequencies to fusidic acid were higher than those to rifampicin with RN4220Δ*mutS*, RN4220 and ST/03/2121 seeing seven-fold and eight-fold increases in mutation frequencies.

The highest mutation frequency to teicoplanin was for the hypermutable isolate, RN4220Δ*mutS* (1.5 x 10^{-6}) (Appendix B, Table 47). This was 20-fold and 22-fold higher than those for RN4220 and ST/03/2121, respectively. However, looking at the mutation frequencies for RN4220Δ*mutS* among experiments, there was a 68-fold difference between the highest and lowest mutation frequencies produced. A similar finding occurred with mutation frequencies to vancomycin, with

the hypermutable isolate showing mutation frequencies 222-fold higher than for RN4220 and 559-fold higher than for ST/03/2121 (Appendix B, Table 48). The mutation frequencies to vancomycin for RN4220Δ*mutS* ranged immensely, from 6.48 x 10^{-6} to 4.17 x 10^{-9}. Mutation frequencies to teicoplanin and vancomycin proved difficult to estimate due to the clustering of colonies on the antibiotic plates. No detectable mutation frequencies were calculable for RN4220, RN4220Δ*mutS*, ST/03/2121 or ST/03/2122 to linezolid with or without the addition of a sub-inhibitory concentration of linezolid in broth prior to plating on linezolid (Appendix B, Table 49).

RN4220Δ*mutS* showed high rates of mutation to resistance to all antibiotics, with ratios above its parent, RN4220, of 11-, 10-, 20- and 222-fold and ST/03/2121 of 9-, 7-, 22- and 560-fold, respectively to rifampicin, fusidic acid, teicoplanin and vancomycin. All mutants selected underwent PFGE to confirm parentage (Figure 16).

3.1.4 Population analysis of mutants

Mutants selected from the mutation frequency experiments, that had been exposed to sub-inhibitory concentrations of vancomycin in broth or plated on agar containing 6 mg/L of vancomycin, underwent population analysis to identify heterogeneous antibiotic resistance, a common occurrence with vancomycin resistant strains, which is only detectable by population analysis (Table 14) (see section 2.3.4). Hetero-resistance was defined by sub-populations with vancomycin MICs between 5 and 8 mg/L. Population analysis revealed six of 13 mutants, generated from mutation frequency experiments, with intermediate-resistance to vancomycin, however, hetero-resistant mutants of ST/03/2122 inherited the trait from their parent strain, i.e., it did not emerge as a result of selection by vancomycin (2 x RN4220Δ*mutS* mutants, 4 x ST/03/2122 mutants and the parent strain ST/03/2122, MICs 5-7 mg/L) (Figure 17, Figure 18 and Figure 19). Vancomycin-selected mutants of RN4220Δ*mutS* were convincingly more resistant than its parent, RN4220, vancomycin-selected variants, with MICs for the latter only reaching a maximum of 3 mg/L (Figure 17 and Figure 18).

Figure 16. PFGE of rifampicin-selected mutants of RN4220Δ*mutS* to confirm parentage.

Lane 12 48.5 Kb lambda ladder, lane 1 RN4220Δ*mutS* control strain, lanes 2-11 RN4220Δ*mutS* rifampicin-selected mutants.

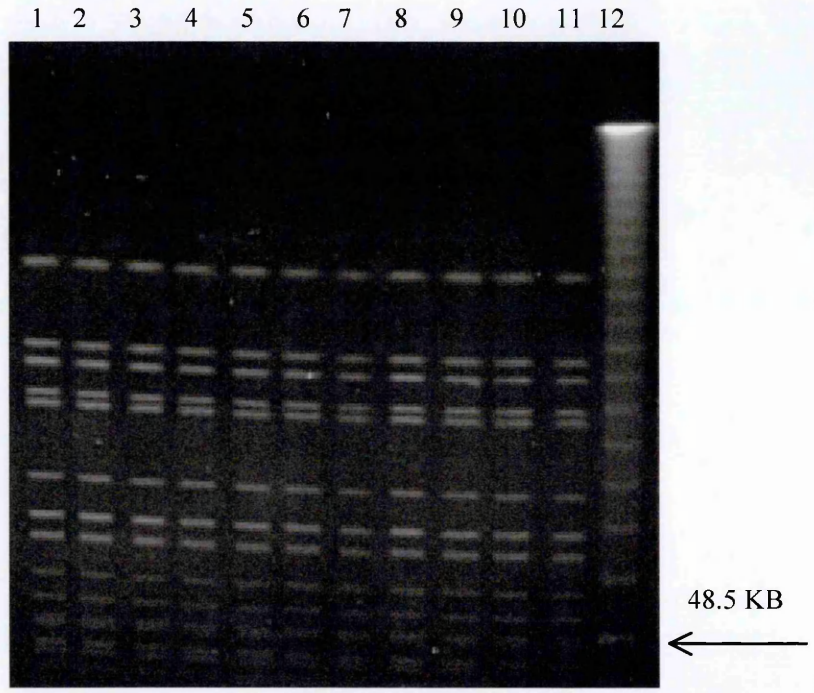


Table 14. Vancomycin MICs of mutants by E Test and population analysis.

Mutant	Selected in broth containing	Selected on agar containing	Vanc MIC (E Test) (mg/L)	Vanc MIC (by pop analysis) (mg/L)
RN4220	N/A	N/A	2	3
RN4220 A	0.25 mg/L cip, 0.5 mg/L vanc	rif (50 mg/L)	2	3
RN4220 B	N/A	vanc (6 mg/L)	4	3
RN4220Δ*mutS*	N/A	N/A	2	3
RN4220Δ*mutS* A	N/A	vanc (6 mg/L)	2	3
RN4220Δ*mutS* B	0.25 mg/L cip, 0.5 mg/L vanc	rif (50 mg/L)	4	5 (heteroVISA)
RN4220Δ*mutS* C	0.25 mg/L cip, 0.5 mg/L vanc	rif (50 mg/L)	4	4
RN4220Δ*mutS* D	0.25 mg/L cip, 0.5 mg/L vanc	rif (50 mg/L)	2	4
RN4220Δ*mutS* E	N/A	vanc (6 mg/L)	2	5 (heteroVISA)
ST/03/2121	N/A	N/A	1	2
ST/03/2121 A	N/A	vanc (6 mg/L)	4	3
ST/03/2121 B	0.25 mg/L cip, 0.5 mg/L vanc	rif (50 mg/L)	4	3
ST/03/2122	N/A	N/A	4	5 (heteroVISA)
ST/03/2122 A	N/A	vanc (6 mg/L)	4	7 (heteroVISA)
ST/03/2122 B	N/A	vanc (6 mg/L)	4	5 (heteroVISA)
ST/03/2122 C	N/A	vanc (6 mg/L)	4	6 (heteroVISA)
ST/03/2122 D	0.25 mg/L cip, 0.5 mg/L vanc	rif (50 mg/L)	8	6 (heteroVISA)

Figure 17. Population analysis of RN4220Δ*mutS* mutants previously exposed to vancomycin.

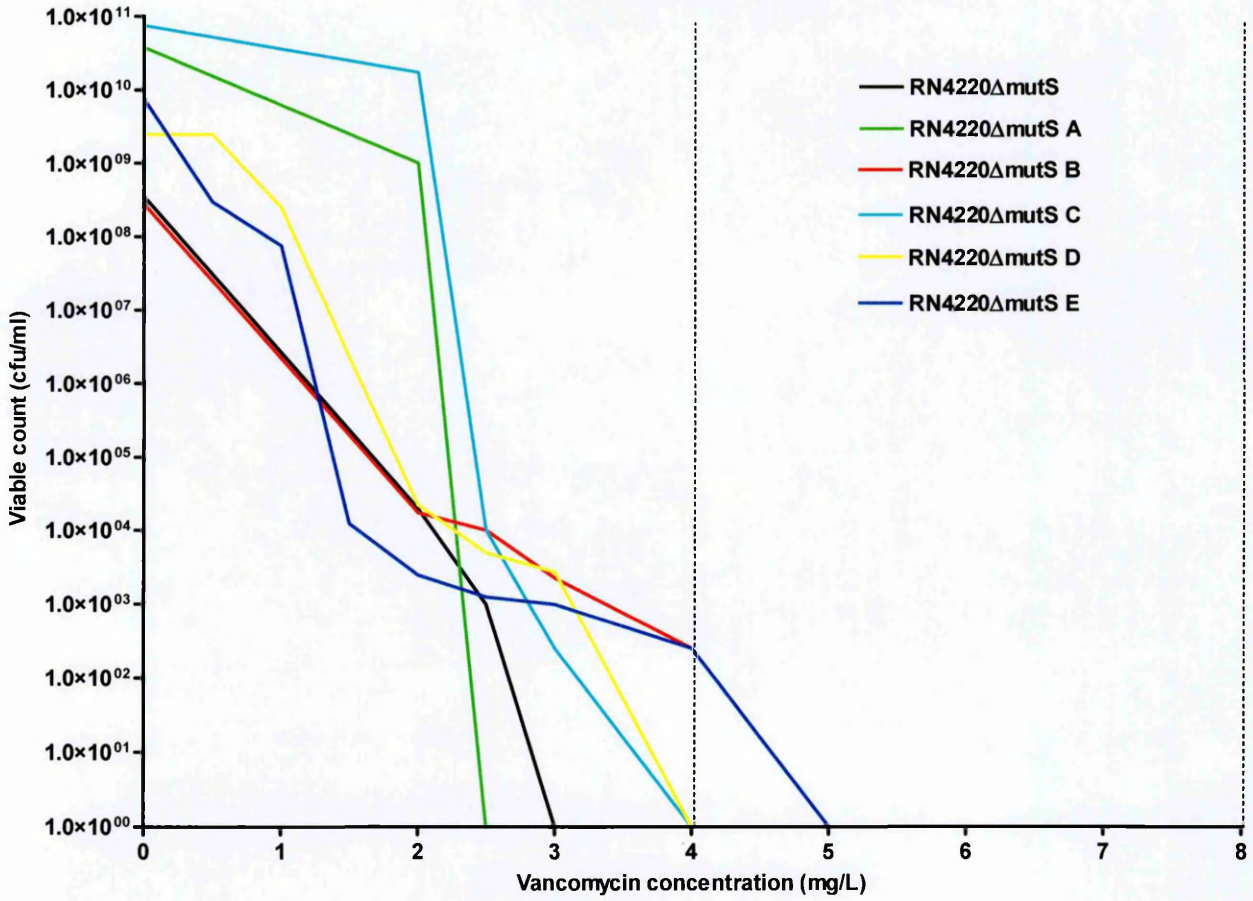


Figure 18. Population analysis of RN4220 mutants previously exposed to vancomycin.

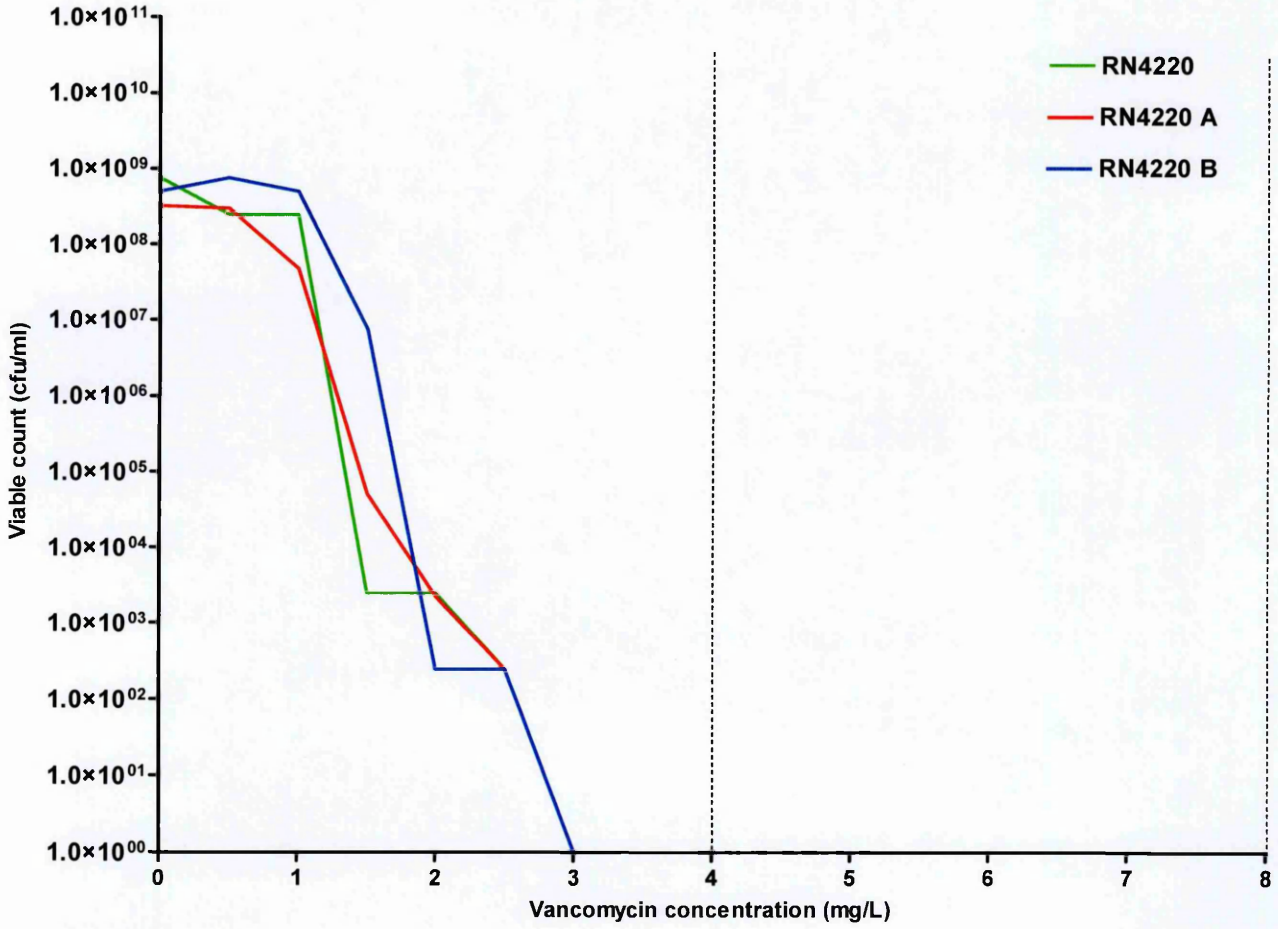
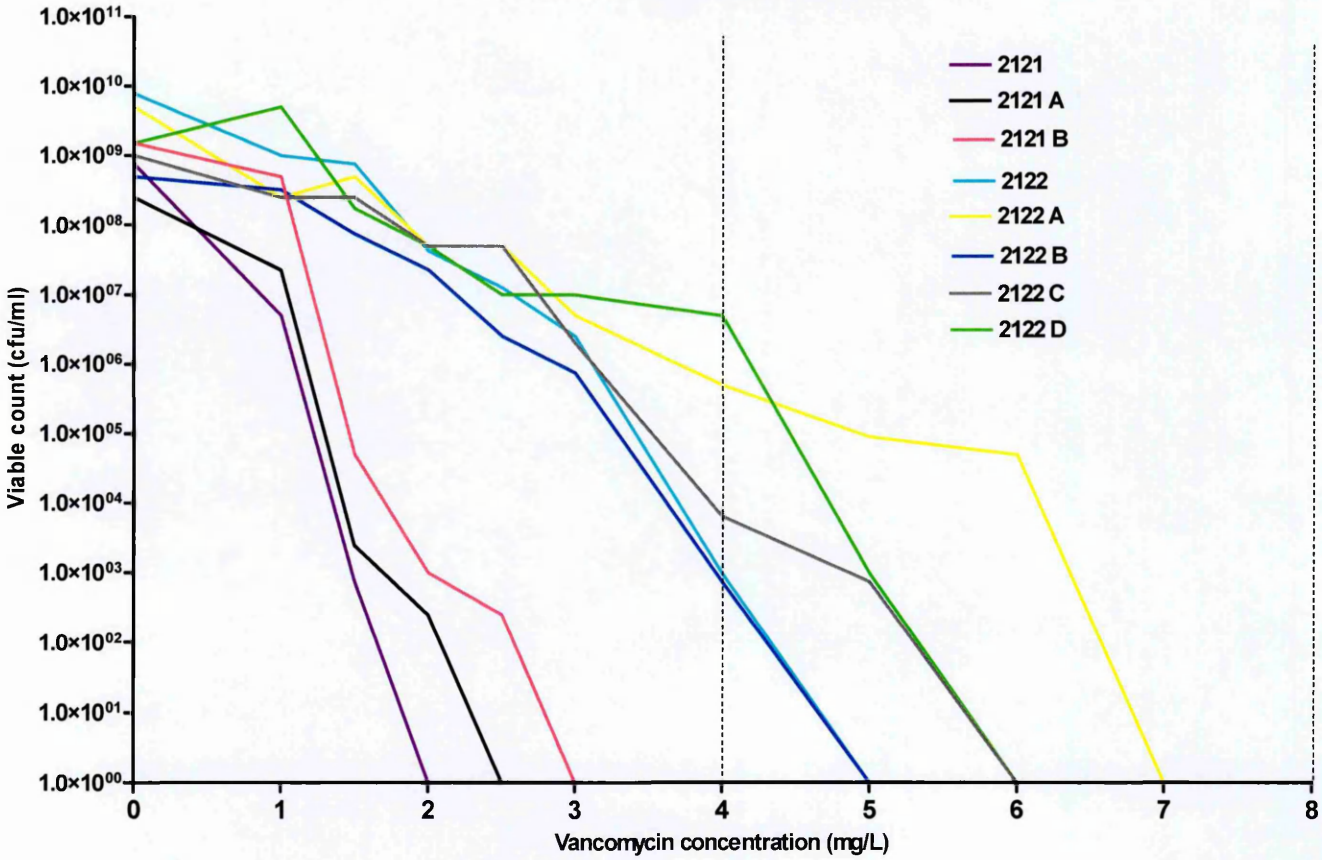


Figure 19. Population analysis of ST/03/2121 and ST/03/2122 mutants previously exposed to vancomycin.



3.1.5 Teicoplanin-resistant mutants

Strain NCTC 8325 and its descendants, RN4220 and RN4220Δ*mutS*, are colourless (Bischoff and Berger-Bachi, 2001). However, when looking at the mutants of RN4220 and RN4220Δ*mutS* selected by exposure to teicoplanin, it was noted that some appeared orange. Mutants selected with other antibiotics did not show this colour change. These mutants consistently appeared after incubation in the presence of teicoplanin (6 mg/L) for a minimum of 48 hours. One orange and one colourless mutant of each of the two strains were investigated further. Teicoplanin MICs for the four mutants, by E Test on Iso agar, ranged from 2-4 mg/L, no different to their parent strains (Table 15) (see section 2.3.3). Additionally, no difference in MIC was observed between the two colony types. Colonies reverted to colourless after serial passage on antibiotic-free agar. The two colony types underwent ten passages, on BHI agar containing 6 mg/L teicoplanin. Serial passage of the orange colony type resulted in a predominance of orange colonies, whereas passage of the colourless colony type resulted in the majority appearing colourless, but also gave rise to a few orange colonies. Teicoplanin MICs, by E Test, after serial passage ranged from 4-8 mg/L, with no obvious differences in MIC between the two colony types. Population analysis revealed parent strains RN4220 and RN4220Δ*mutS* with teicoplanin MICs of 3 mg/L and 5 mg/L, respectively, and all mutants with MICs of >8 mg/L (Figure 20). PFGE confirmed parentage of both colony types before and after passaging. No differences in the PFGE profiles of mutants were observed.

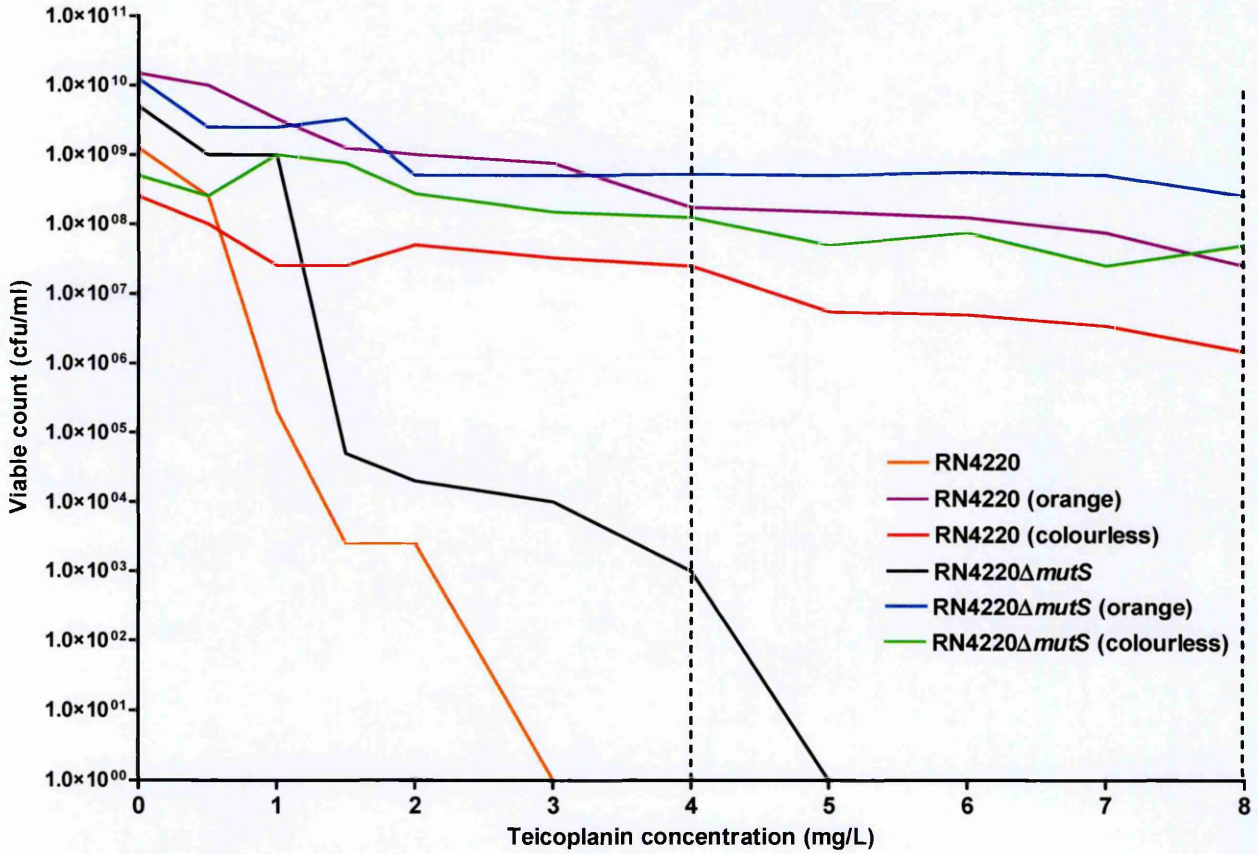
3.1.6 Summary

The wild-type and hypermutable reference strains, RN4220Δ*mutS* and RN4220, differed in their PFGE profiles due to the integration of the pGhost9^{+} plasmid, with an extra *Sma*I cutting site, in the *mutS* gene of the hypermutable strain. The insertion of the plasmid also resulted in the introduction of an *erm*(B) determinant into the hypermutable strain. These differences enabled differentiation of RN4220 and RN4220Δ*mutS* and their mutants. Mutation frequencies of these two strains along with two clinical isolates confirmed that the hypermutable strain had elevated mutation frequencies to a range of antibiotics when compared with its wild-type parent strain or with clinical isolates.

Table 15. Mutants of RN4220 and RN4220Δ*mutS* obtained under teicoplanin selection.

	Teicoplanin MIC (mg/L)		
Clone	**Before serial passage**	**After serial passage**	**After population analysis**
RN4220	**2**	**N/A**	**3**
RN4220-orange	2	4	>8
RN4220-colourless	2	4	>8
RN4220Δ*muts*	**4**	**N/A**	**5**
RN4220Δ*muts*-orange	4	8	>8
RN4220Δ*muts*-colourless	4	4	>8

Figure 20. Population analysis of teicoplanin-resistant mutants of RN4220 and RN4220Δ*mutS*.



Mutation frequencies to teicoplanin and vancomycin proved difficult to calculate due to the clustering of colonies on antibiotic-containing plates, making counting difficult and resulting in a wide range of mutation frequencies being found. Population analysis of the mutants exposed to vancomycin during mutation frequency experiments revealed that six of 13 mutants selected had hetero-resistance to vancomycin, but that only two emerged as heteroresistant during the experiment. Two colony types were generated from mutation frequency experiments to teicoplanin and serial passage in the presence of teicoplanin stabilized these mutant strains. Population analysis revealed the mutant strains with teicoplanin MICs in excess of 8 mg/L. No mutation frequencies to linezolid were determined as no mutants were obtained.

3.2 Laboratory-selected linezolid-resistant mutants

No mutants were selected in the single-step linezolid mutation frequency experiments; therefore to obtain linezolid-resistant mutants a change in methodology was adopted. Linezolid-resistant variants were selected by exposure to increasing concentrations of linezolid in broth or on antibiotic plates (see section 2.5.1).

3.2.1 Raising linezolid-resistant mutants *in vitro*

Once mutants were able to grow in broth containing 10 mg/L linezolid they were transferred to BHI agar containing 6 mg/L linezolid (see section 2.5.1). Linezolid E Tests revealed these mutants with MICs of 4 or 8 mg/L (see section 2.3.3). The mutants were then passaged five times on BHI agar containing 10 mg/L of linezolid to try to stabilize resistance. After serial passage however, linezolid MICs of some mutants fell to 4-8 mg/L, implying selected resistance was unstable. However the variations in MIC might also be attributed to the medium used; mutants were selected on BHI agar but MICs were determined on ISO agar.

3.2.2 Characterization of laboratory-selected linezolid-resistant mutants

Seventeen linezolid-resistant mutants of RN4220, RN4220Δ*mutS* and ST/03/2121 were selected *in vitro*; no mutants of ST/03/2122 were raised (Table 16). Firstly, in order to characterize the mutants, linezolid MICs were determined (Table 16) (see section 2.3). Secondly, all mutants were screened for a G2576T mutation by PCR-RFLP with the restriction endonuclease *Nhe*I (see section 2.7.4) (Woodford *et al.*, 2002). A 694 bp fragment of the 23S rRNA genes was amplified and then digested with *Nhe*I (Figure 21 and Figure 22). Next, the same 694 bp amplicon of the 23S rRNA genes was amplified and DNA sequenced for all mutants (see section 2.7.6). Sequences of the linezolid-resistant isolates were aligned with those of their parents to identify any differences. DNA sequencing confirmed the presence of G2576T, T2504C and G2447T mutations in various mutants (Figure 23 [A], [B] and [C]). Finally, after a T2504C mutation had been identified by sequencing, a *Hin*1I PCR-RFLP was developed to detect the presence of a T to C change at position 2504 and used to screen all mutants (Figure 24) (see section 2.7.4 and 2.7.5).

Linezolid MICs for mutants ranged from 8-64 mg/L. Ten of 17 mutants had a G2447T mutation, four had a G2576T mutation, and one mutant had a T2504C mutation. The G2576T mutation was seen only in mutants of RN4220Δ*mutS*. All mutants were heterozygous for their respective mutations. All mutants had a single mutation type. Two mutants with MICs 8 and 32 mg/L had unidentifiable mechanisms of resistance. Parentage of mutants was confirmed by PFGE (see section 2.6.3).

Pyrosequencing, with three primers, was used to detect the presence of seven mutations already known to confer resistance to linezolid; T2500A, A2503G, T2504C, G2505A, G2445T, G2447T and G2576T (see sections 2.7.1 and 2.7.7). Three replicate pyrosequencing experiments were performed for each isolate for each sequencing primer and an average of the percentage mutated and wild-type 23S rRNA gene copies for each mutation was calculated (Table 17). Pyrosequencing detected mutations in all but two of the mutants and allowed quantification of mutated 23S rRNA gene copies in six mutants. Pyrosequencing results for identification of mutation type and heterozygosity were in agreement with PCR-RFLP and sequencing results, with

Table 16. MICs for laboratory-selected linezolid-resistant mutants.

Clone	Mutation	Lin[A] MIC (mg/L)	Chlor[B] MIC (mg/L)
RN4220	**Control**	**2**	**4**
RN4220-M1	G2447T	32	4
RN4220-M2	G2447T	8	4
RN4220-M3	G2447T	32	4
RN4220Δ*mutS*	**Control**	**2**	**4**
RN4220Δ*mutS*-M4	G2447T	32	4
RN4220Δ*mutS*-M5	G2447T	16	4
RN4220Δ*mutS*-M6	G2447T	32	4
RN4220Δ*mutS*-M7	G2576T	32	8
RN4220Δ*mutS*-M8	G2576T	32	32
RN4220Δ*mutS*-M9	G2576T	32	32
RN4220Δ*mutS*-M10	G2576T	16	32
ST/03/2121	**Control**	**4**	**8**
ST/03/2121-M11	T2504C	64	32
ST/03/2121-M12	G2447T	16	8
ST/03/2121-M13	G2447T	16	8
ST/03/2121-M14	G2447T	16	8
ST/03/2121-M15	G2447T	32	8
ST/03/2121-M16	Unknown mutation	8	4
ST/03/2121-M17	Unknown mutation	32	8
ST/03/2122	**Control**	**1**	**2**

[A]Linezolid, [B]Chloramphenicol.

Figure 21. PCR to amplify a 694 bp fragment of the 23S rRNA genes. Lanes 1 and 9 123 bp ladder, lanes 2-7 694 bp PCR product, lane 8 water control.

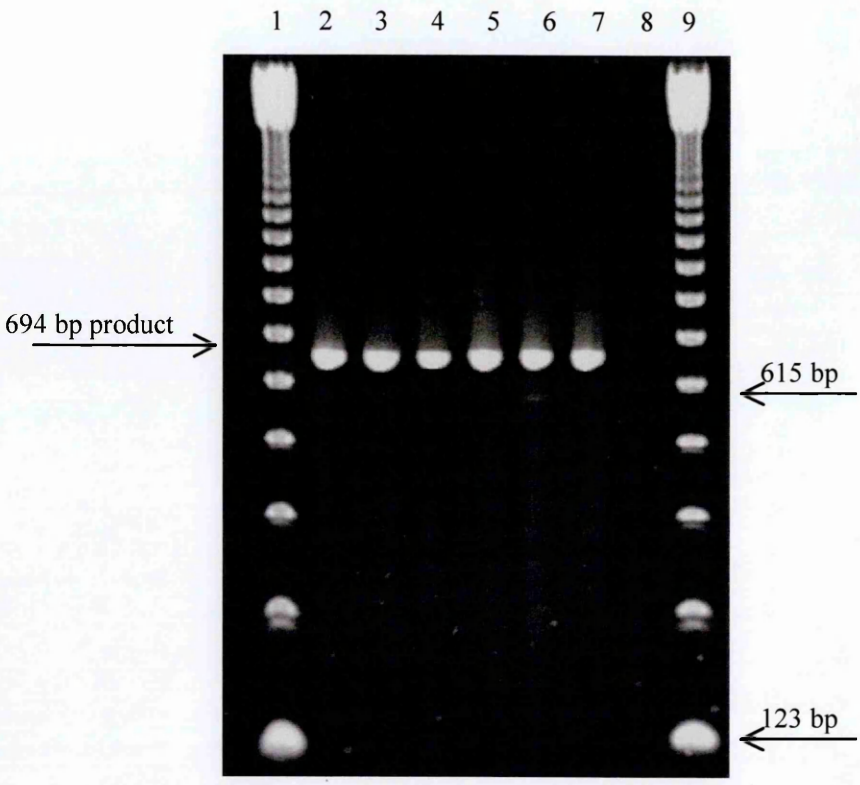


Figure 22. Digestion of a 694 bp amplicon of the 23S rRNA genes with the restriction enzyme *Nhe*I to determine the presence of a G2576T mutation conferring linezolid resistance. Lane 1 homozygous T (all 23S rRNA copies mutated) lane 2 homozygous G (wild type, no copies mutated), lane 3 heterozygous T/G (some mutated copies and some wild-type copies), lane 4 123 bp ladder.

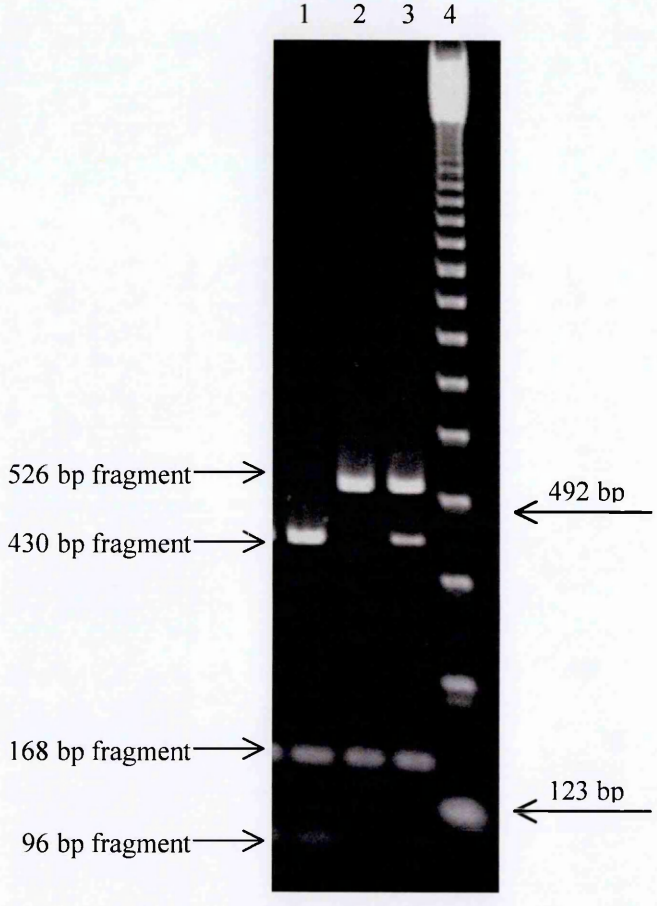


Figure 23. DNA sequencing chromatograms of linezolid-resistant mutants. **(A)** RN4220Δ*mutS*-M7 with a G2576T mutation. The red 'T' peak under the black 'G' peak indicates heterozygous resistance. **(B)** Chromatogram of ST/03/2121-M11 with a T2504C mutation. The blue 'C' peak under the red 'T' peak indicates heterozygous resistance. **(C)** Chromatogram of RN4220-M1 with a G2447T mutation. The red 'T' peak under the black 'G' peak indicates heterozygous resistance.

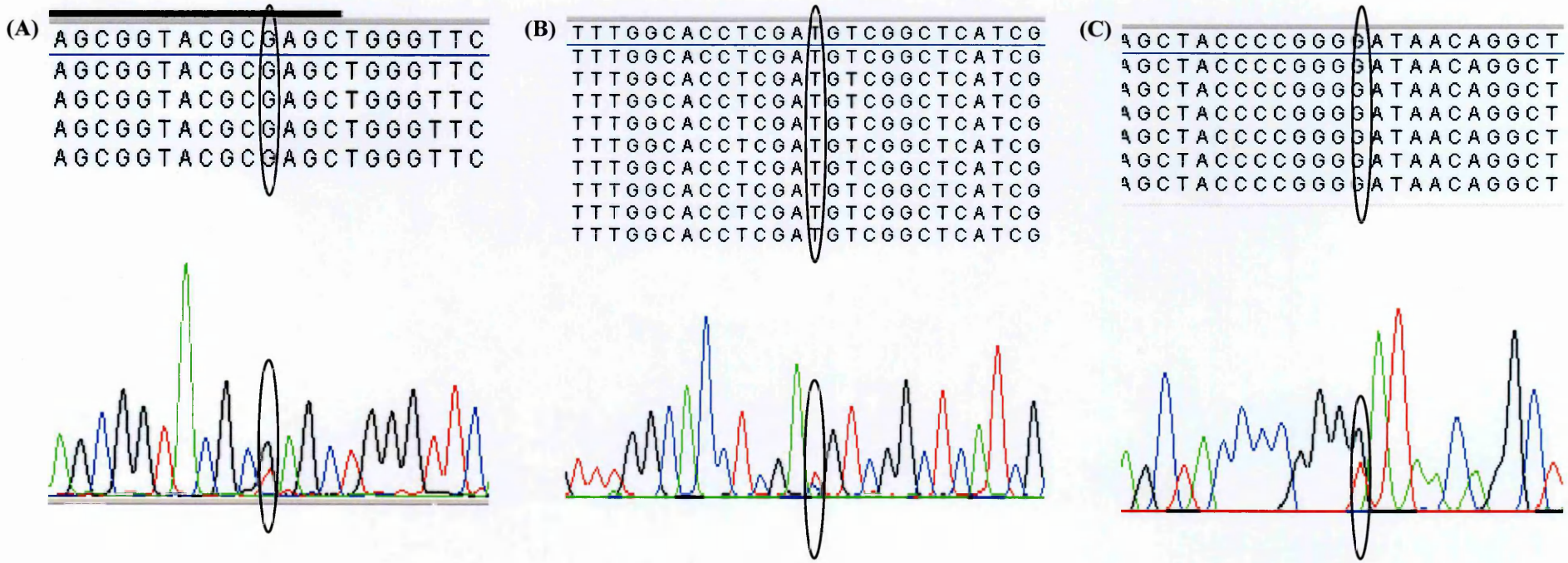


Figure 24. Digestion of a 694 bp amplicon of the 23S rRNA genes with the restriction enzyme *Hin*1I, to determine the presence of a G2576T mutation conferring linezolid resistance. Lane 1 123 bp ladder, lane 2 homozygous T (no 23S rRNA copies mutated), lane 3 heterozygous T/C (some wild-type copies and some mutated copies).

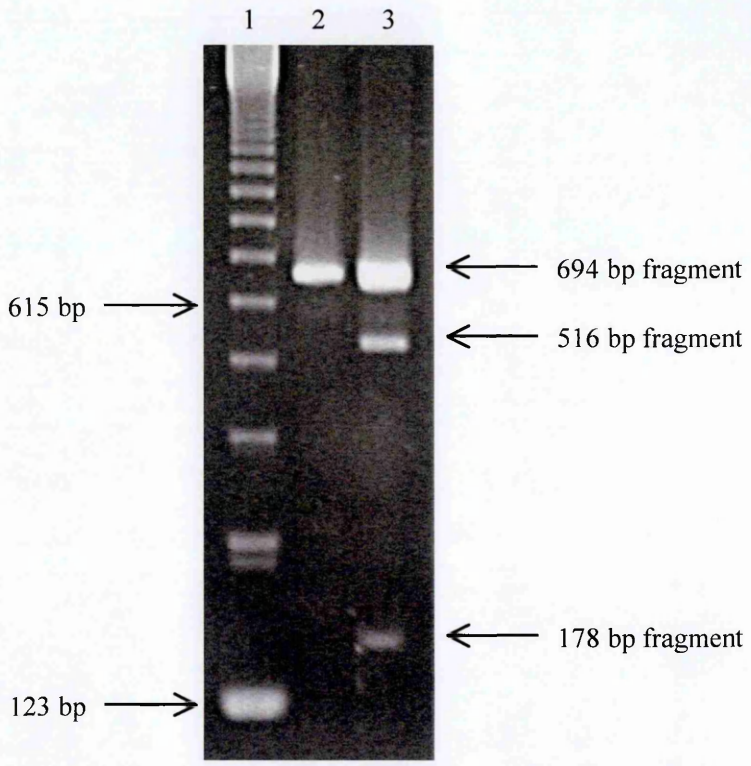


Table 17. Pyrosequencing and hybridization results for laboratory-selected linezolid-resistant mutants. Numbers in brackets refer to increase or decrease in total 23S rRNA gene copy number when compared with parent strains. (See Appendix B Table 55 for a complete summary of pyrosequencing and hybridization results)

Strain	Mutation	Lin MIC (mg/L)	23S rRNA gene copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WT copies
RN4220	**N/A**	**4**	**6**	**WT[A]**	**WT**	**WT**
RN4220-M1	G2447T	16	6	N/A [B]	N/A [B]	N/A [B]
RN4220-M2	G2447T	8	6	N/A [B]	N/A [B]	N/A [B]
RN4220-M3	G2447T	16	6	N/A [B]	N/A [B]	N/A [B]
RN4220Δ*mutS*	**N/A**	**4**	**6**	**WT**	**WT**	**WT**
RN4220Δ*mutS* -M4	G2447T	16	5 (-1)	N/A [B]	N/A [B]	N/A [B]
RN4220Δ*mutS* -M5	G2447T	16	6	N/A [B]	N/A [B]	N/A [B]
RN4220Δ*mutS* -M6	G2447T	16	6	N/A [B]	N/A [B]	N/A [B]
RN4220Δ*mutS*-M7	G2576T	16	6	33.3	66.7	2:4
RN4220Δ*mutS*-M8	G2576T	16	6	31.9	68.1	2:4
RN4220Δ*mutS*-M9	G2576T	16	6	45.0	55.0	3:3
RN4220Δ*mutS*-M10	G2576T	16	6	48.9	51.1	3:3
ST/03/2121	**N/A**	**4**	**5**	**WT**	**WT**	**WT**
ST/03/2121-M11	T2504C	16	8 (+3)	38.0	62.0	3:5

Cont.

Table 17. Pyrosequencing and hybridization results for laboratory-selected linezolid-resistant mutants (cont.). (See Appendix B Table 55 for a complete summary of pyrosequencing and hybridization results).

Strain	Mutation	Lin MIC (mg/L)	23S rRNA gene copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WT copies
ST/03/2121-M12	G2447T	16	6 (+1)	N/A [B]	N/A [B]	N/A [B]
ST/03/2121-M13	G2447T	16	5	N/A [B]	N/A [B]	N/A [A]
ST/03/2121-M14	G2447T	16	6 (+1)	N/A [B]	N/A [B]	N/A [A]
ST/03/2121-M15	G2447T	16	4 (-1)	N/A [B]	N/A [B]	N/A [A]
ST/03/2121-M16	Unknown mutation	8	5	N/A	N/A	N/A
ST/03/2121-M17	Unknown mutation	16	7 (+2)	N/A	N/A	N/A

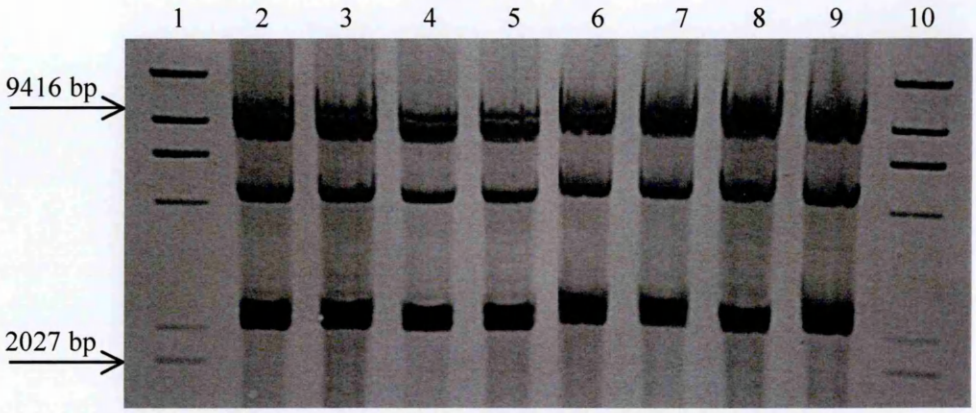
[A] Wild-type (WT) [B] Quantification of the percentage of mutated copies was not possible for G2447T mutations due to the mutation residing within a homopolymer of G nucleotides.

all three techniques detecting the same mutations. Five mutants, all with linezolid MICs of 16 mg/L and the G2576T mutation, had between 32% and 49% of copies mutated. All five mutants had the same number of genes encoding 23S rRNA copies as their parent strains. One mutant, with a T2504C mutation, had three of eight (38%) gene copies mutated. Identification of the resistance mechanism of two mutants (ST/03/2121-M16, ST/03/2121-M17) could not be elucidated using these methods.

More generally, no mutants with a G2445T mutation were detected, meaning that the assay could not be verified as detecting this mutation. Mutations G2445T and G2447T are present within a homopolymer of G nucleotides and, whilst detection of these mutations was possible (or theoretically possible in the case of G2445T), the pyrosequencing software did not allow quantification (see section 3.6 for more details on the use of pyrosequencing and hybridization to determine the number of mutated 23S rRNA gene copies).

Hybridization was used to determine 23S rRNA gene copy number (see section 2.8). The results of the hybridization and pyrosequencing were combined to calculate the actual number of mutated 23S rRNA gene copies (Table 17). Linezolid MICs for the mutants were repeated shortly before hybridization and pyrosequencing were performed, and slight decreases in linezolid MIC were noted when compared with previous values. RN4220 and RN4220Δ*mutS* had six gene copies and ST/03/2121 had five gene copies encoding 23S rRNA (Figure 25), whereas the 23S rRNA gene copy number in their mutants varied from four to eight. Six mutants had changes in gene copy number when compared with their parent strains; specifically one RN4220 mutant (M4) and five ST/03/2121 mutants (M11, M12, M14, M15, M16, M17). In four of the six mutants the 23S rRNA gene copy number increased or decreased by one whereas mutants M11 and M17 had increases in 23S rRNA gene copy number by two and three copies, respectively. Four of the six mutants with changes in 23S rRNA gene copy number had G2447T mutations (M4, M12, M14, M15), one had a T2504C mutation (M11); one had no detectable mechanism of resistance (M17). Linezolid MICs for those mutants with changes in 23S rRNA gene copy number were all 16 mg/L. The four mutants with a G2576T mutation did not have any changes in gene copy number.

Figure 25. Hybridization of DNA from linezolid-resistant mutants of RN4220 and RN4220Δ*mutS* with a 420 bp 23S rRNA gene probe. Lanes 1 and 10 DIG-labelled ladder, lane 2 RN4220, lanes 3-5 RN4220-M1-M3, lane 6 RN4220Δ*mutS*, lanes 7-9 RN4220Δ*mutS*-M7-M9.



One mutant with a T2504C mutation (M11) showed a four-fold increase in chloramphenicol MIC from 8 to 32 mg/L (Table 16), whilst eight-fold increases in chloramphenicol MIC were observed in three out of the four mutants with G2576T mutations (MICs rose from 4 to 32 mg/L in mutants M8, M9, M10). However, one mutant with a G2576T mutation only had a small increase in chloramphenicol MIC from 4 to 8 mg/L (M7).

3.2.3 Reversion to wild type of linezolid-resistant RN4220Δ*mutS* mutants

Further characterization of the *in-vitro*-selected linezolid-resistant mutants revealed some RN4220Δ*mutS* mutants to be erythromycin-susceptible (Table 18), whereas their parent strain, RN4220Δ*mutS*, had an erythromycin marker, *erm*(B), on the plasmid inserted to disrupt the *mutS* gene and, consequently, had an erythromycin MIC >256 mg/L. PFGE confirmed reversion to a 'wild-type' RN4220 profile in five of seven RN4220Δ*mutS* linezolid-resistant mutants, with loss of the pGhost9$^+$ plasmid (Figure 26). Long PCR of the *mutS* gene was used to confirm the presence or absence of the pGhost9$^+$ plasmid and to determine whether complete excision had taken place (Figure 27) (see sections 2.6.1 and 2.6.2). PCR of the *mutS* gene in RN4220 produces a 1.5 Kb product, whereas PCR of *mutS* in RN4220Δ*mutS* produces a PCR product of 6.1 Kb, due to the presence of the plasmid. RN4220Δ*mutS* variants from which the plasmid has been excised should produce a 1.5 Kb PCR product, as with RN4220. Five of seven RN4220Δ*mutS* linezolid-resistant mutants did this, with seemingly complete excision of the plasmid leaving an intact, restored *mutS* gene. Long PCR also revealed that, in RN4220Δ*mutS*, and its erythromycin-resistant mutants, some excision of the plasmid was occurring, indicated by both 1.5 Kb and 6.1 Kb PCR products being produced. Subsequent to this discovery, RN4220Δ*mutS* and its mutants were grown at 42°C to force integration of the thermosensitive plasmid or in the presence of an erythromycin disc (30 μg) to prevent reversion to wild type (O'Neill and Chopra, 2002) (see section 2.4.1).

Table 18. Characteristics of laboratory-selected linezolid-resistant mutants.

Clone	Mutation	Characteristics	Lin MIC (mg/L)	Ery MIC (mg/L)
RN4220	**Control**	**N/A**	**2**	**1**
RN4220-M1	G2447T	N/A	32	1
RN4220-M2	G2447T	N/A	8	1
RN4220-M3	G2447T	N/A	32	1
RN4220Δ*mutS*	**Control**	**Δ*mutS***	**2**	**>256**
RN4220Δ*mutS*-M4	G2447T	*mutS* (revertant)	32	1
RN4220Δ*mutS*-M5	G2447T	*mutS* (revertant)	16	1
RN4220Δ*mutS*-M6	G2447T	*mutS* (revertant)	32	1
RN4220Δ*mutS*-M7	G2576T	*mutS* (revertant)	32	1
RN4220Δ*mutS*-M8	G2576T	*mutS* (revertant)	32	1
RN4220Δ*mutS*-M9	G2576T	Δ*mutS*	32	>256
RN4220Δ*mutS*-M10	G2576T	Δ*mutS*	16	>256
ST/03/2121	**Control**	**N/A**	**4**	**>256** [A]
ST/03/2121-M11	T2504C	N/A	64	>256 [A]
ST/03/2121-M12	G2447T	N/A	16	>256 [A]
ST/03/2121-M13	G2447T	N/A	16	>256 [A]
ST/03/2121-M14	G2447T	N/A	16	>256 [A]
ST/03/2121-M15	G2447T	N/A	32	>256 [A]
ST/03/2121-M16	Unknown mutation	N/A	8	>256 [A]
ST/03/2121-M17	Unknown mutation	N/A	32	>256 [A]
ST/03/2122	**Control**	**N/A**	**1**	**>256** [A]

[A] Parent strains ST/03/2121 and ST/03/2122 and their mutants all have an *erm*(C) gene, conferring resistance to erythromycin.

Figure 26. PFGE of *Sma*I-digested DNA from laboratory-selected linezolid-resistant mutants. Lanes 1 and 22 48.5 Kb lambda ladder, lane 2 RN4220 control, lanes 3-5 RN4220 mutants M1-M3, lane 6 RN4220Δ*mutS* control, lanes 7-13 RN4220Δ*mutS* mutants M4-M10, lane 14 ST/03/2121 control, lanes 15-21 ST/03/2121 mutants M11-M17. Note that mutants M4-M8 (lanes 7-11) have regained the profile of wild-type RN4220 (lane 2).

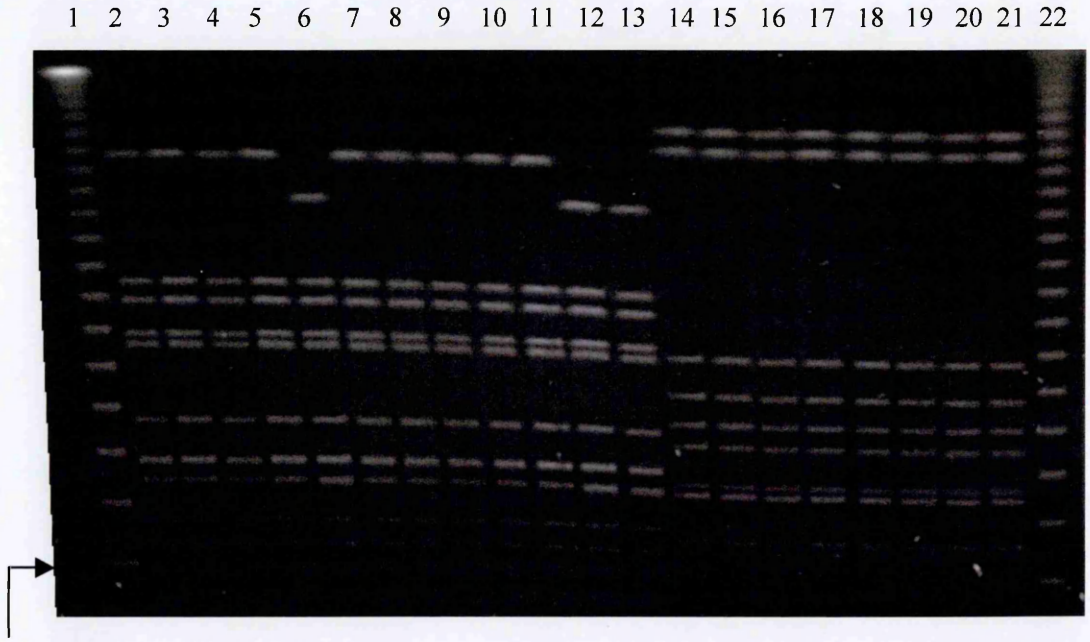
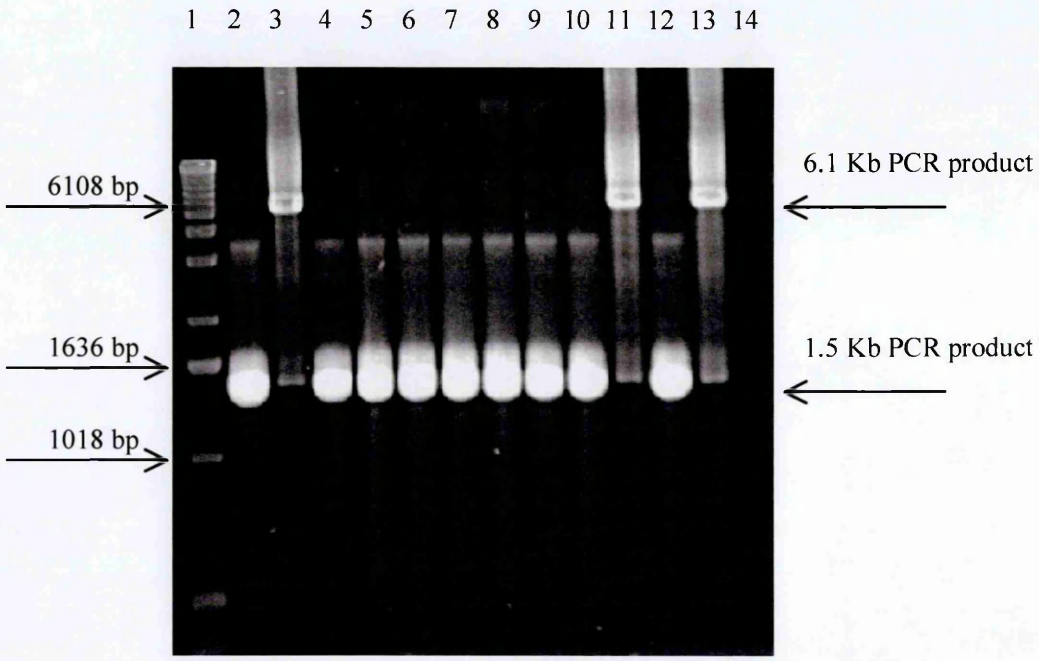


Figure 27. Long PCR of laboratory-selected linezolid-resistant mutants of RN4220Δ*mutS*. Lane 1 1 Kb ladder, lane 2 RN4220 control, lane 3 RN4220Δ*mutS* control, lanes 4-10 RN4220Δ*mutS* mutants M4-M7 (revertants), lane 11 RN4220Δ*mutS* mutant-M9, lane 12 RN4220Δ*mutS* mutant-M8 (revertant), lane 13 RN4220Δ*mutS* mutant-M10, lane 14 water control.



3.2.4 Raising a linezolid-resistant mutant homozygous for T2576

This experiment was designed to determine whether mutated 23S rRNA gene copy number would increase in direct correlation with linezolid MIC. The resulting mutant was predicted to have a relatively high linezolid MIC and all 23S rRNA gene copies would be mutated to a T at position 2576.

RN4220*mutS*-M9 was heterozygous for the G2576T mutation in its 23S rRNA genes and, in an attempt to generate a mutant homozygous for the G2576T mutation, was passaged on increasing concentrations of linezolid. As well as linezolid, the mutant (now designated RN4220*mutS*-M9a) was grown in the presence of a 30 μg erythromycin disc or with 50 mg/L erythromycin incorporated into agar to prevent reversion to wild-type. The mutant was passaged over the course of four months on BHI agar containing increasing concentrations of linezolid (in 2 mg/L increments from 16-300 mg/L). The mutant was passaged at the same concentration of linezolid until it had grown adequately before being passaged at a higher concentration. The mutant was tested at fortnightly intervals to see whether all 23S rRNA gene copies had mutated, by digesting a 694 bp PCR product with the restriction endonuclease *Nhe*I (Woodford *et al.*, 2002). All *Nhe*I digests nevertheless remained heterozygous for G2576T. The mutant linezolid MIC was >256 mg/L by E Test but resistance was unstable and some reversion towards linezolid susceptibility occurred if the mutant was passaged on antibiotic-free agar. DNA sequencing of a 694 bp region of the 23S rRNA genes confirmed the presence of a G2576T mutation (Figure 28 [A]). More 23S rRNA gene copies were mutated (T) than wild-type (G), as indicated by peak heights on the chromatogram. Additionally, an adenine to guanine change at position 2503 was detected (Figure 28 [B]). Pyrosequencing detected and determined the percentage of mutated 23S rRNA gene copies for the two mutations (Table 19). For the A2503G mutation 32% of copies were mutated and for the G2576T mutation 64% of copies were mutated, compared to 45% in the parent strain. Hybridization estimated the number of 23S rRNA copies present, and using both pyrosequencing and hybridization, the number of mutated copies was determined. Hybridization confirmed the presence of six 23S rRNA gene copies in both the parent and mutant strains. For the

Figure 28. Chromatogram of G2576T and A2503G mutations in laboratory-selected linezolid-resistant mutant RN4220Δ*mutS*-M9a. **(A)** G2576T mutation. The presence of a black 'G' peak under the red 'T' peak indicates heterozygous resistance. The sequencing software is calling a 'T', indicating an increase and predominance in the number of mutated copies after passage on increasing concentrations of linezolid. **(B)** A2503G mutation. The presence of a black 'G' peak under a green 'A' peak indicates heterozygosity.

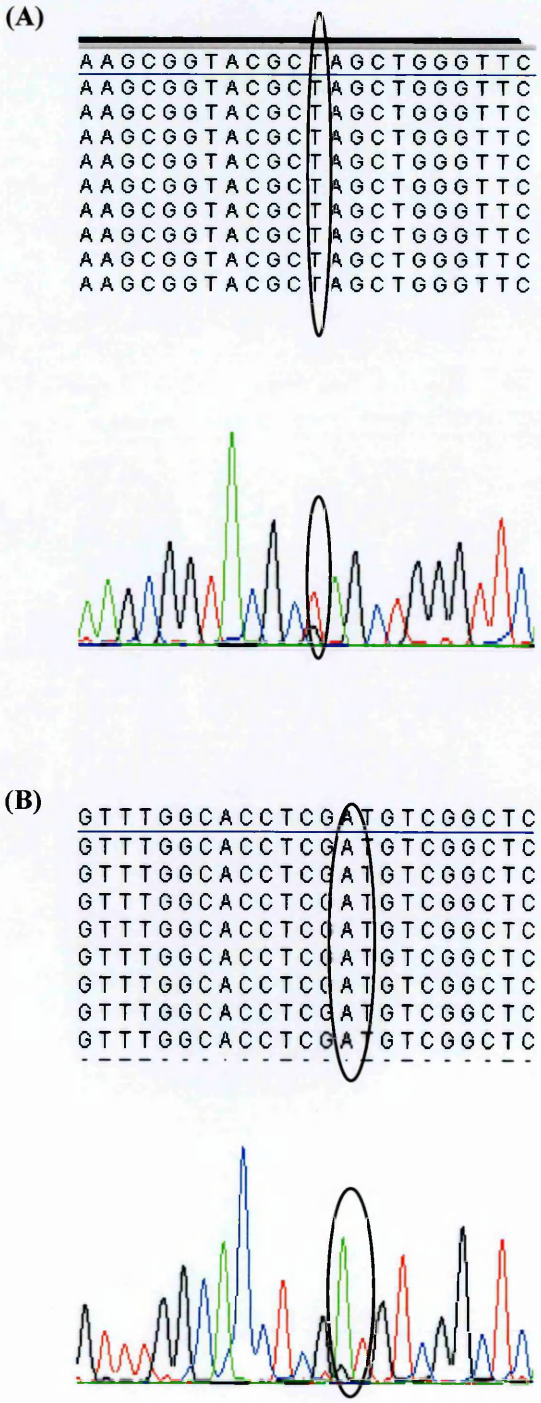


Table 19. Pyrosequencing and hybridization results for control strain RN4220Δ*mutS* and laboratory-selected linezolid-resistant mutants RN4220Δ*mutS*-M9 and RN4220Δ*mutS*-M9a. (See Appendix B Table 55 for a complete summary of pyrosequencing and hybridization results).

Strain	Lin MIC (mg/L)	Mutation	Average % WT: mutated copies	No. of 23S rRNA copies	No. WT: mutated copies
RN4220Δ*mutS*	4	N/A	N/A	6	N/A
RN4220Δ*mutS*-M9	16	G2576T	45 : 55	6	3 : 3
RN4220Δ*mutS*-M9a	>256	G2503A	68 : 32	6	4 : 2
		G2576T	36 : 64	6	2 : 4

A2503G mutation, two gene copies were mutated and for the G2576T mutation, four gene copies were mutated. Using this technique it is not possible to determine which of the gene copies were mutated. All six gene copies might have one mutation present, or four gene copies could be mutated with two of these carrying both G2576T and A2503G mutations. PFGE confirmed parentage of the mutant.

3.2.5 Mutation frequencies of laboratory-selected linezolid-resistant mutants

In order to determine whether selecting for linezolid resistance altered the mutability of the strains, and co-selected for hypermutability, mutation frequencies of the linezolid-resistant mutants were determined. The frequency at which *in vitro*-selected linezolid-resistant mutants of RN4220, RN4220Δ*mutS* and ST/03/2121 and their parent strains generated mutants to rifampicin (50 mg/L) and fusidic acid (0.5 mg/L) was determined (see Table 20 for MICs) (see section 2.9.1). The arithmetic mean of three mutation frequency experiments was graphed and the mean mutation frequencies represented.

RN4220, RN4220Δ*mutS* and ST/03/2121 controls all had mutation frequencies to rifampicin and fusidic acid comparable with those found in previous experiments (Table 21, Figure 29, Figure 30, Appendix B Table 50 and Table 51). Out of 34 average mutation frequencies to rifampicin and fusidic acid, half increased and half decreased compared with their parent strains. Twelve mutants had increases in mutation frequency to rifampicin, five had decreases in mutation frequencies, when compared with their parent strains. Four mutants had increases in mutation frequency to fusidic acid and 13 had decreases, when compared with their parent strains. Four mutants had increases in average mutation frequencies to both rifampicin and fusidic acid, when compared with their parent strains; two mutants of RN4220 (M1, M2,), one revertant mutant (wild-type, erythromycin-susceptible) of RN4220Δ*mutS* (M6) and one mutant of RN4220Δ*mutS* (M10). Of these four mutants, three had G2447T mutations and one had a G2576T mutation conferring resistance to linezolid. Five mutants had decreases in both rifampicin and fusidic acid mutation frequencies when compared with their parent strains, four of the five were mutants of ST/03/2121

Table 20. MICs for laboratory-selected linezolid-resistant strains to rifampicin and fusidic acid.

Clone	Mutation	Characteristics	Lin MIC (mg/L)	Rif MIC mg/L	Fus MIC mg/L
RN4220	**Control**	**WT**	**2**	**0.016**	**0.125**
RN4220-M1	G2447T	WT	32	0.016	0.25
RN4220-M2	G2447T	WT	8	0.016	0.25
RN4220-M3	G2447T	WT	32	0.016	0.25
RN4220Δ*mutS*	**Control**	**Δ*mutS***	**2**	**0.016**	**0.25**
RN4220Δ*mutS*-M4	G2447T	*mutS* (revertant)	32	0.016	0.25
RN4220Δ*mutS*-M5	G2447T	*mutS* (revertant)	16	0.016	0.25
RN4220Δ*mutS*-M6	G2447T	*mutS* (revertant)	32	0.016	0.25
RN4220Δ*mutS*-M7	G2576T	*mutS* (revertant)	32	0.016	0.25
RN4220Δ*mutS*-M8	G2576T	*mutS* (revertant)	32	0.016	0.25
RN4220Δ*mutS*-M9	G2576T	Δ*mutS*	32	0.016	0.25
RN4220Δ*mutS*-M10	G2576T	Δ*mutS*	16	0.016	0.25
ST/03/2121	**Control**	**WT**	**4**	**0.08**	**0.25**
ST/03/2121-M11	T2504C	WT	64	0.08	0.125
ST/03/2121-M12	G2447T	WT	16	0.08	0.125
ST/03/2121-M13	G2447T	WT	16	0.08	0.125
ST/03/2121-M14	G2447T	WT	16	0.08	0.125

Cont.

Table 20. MICs for laboratory-selected linezolid-resistant strains to rifampicin and fusidic acid (cont.).

ST/03/2121-M15	G2447T	WT	32	0.08	0.125
ST/03/2121-M16	Unknown mutation	WT	8	0.08	0.125
ST/03/2121-M17	Unknown mutation	WT	32	0.08	0.125
ST/03/2122	**Control**	**WT**	**1**	**>256**	**0.064**

Table 21. Summary of mutation frequencies of laboratory-selected mutants to rifampicin and fusidic acid. (See Appendix B Table 50 and Table 51 for more detailed results).

Isolate	23S rRNA Mutation	Characteristics	Av. m freq to rif[A] ± SD[B]	Fold ↑ or ↓[C]	Av. m freq to fus[D] ±SD	Fold ↑ or ↓
RN4220	**Control**	**WT**	**$1.67 \pm 0.67 \times 10^{-8}$**	**WT**	**$2.56 \pm 1.82 \times 10^{-7}$**	**W/T**
RN4220-M1	G2447T	WT	$4.08 \pm 1.46 \times 10^{-8}$	2 fold ↑	$3.94 \pm 0.69 \times 10^{-7}$	2 fold ↑
RN4220-M2	G2447T	WT	$2.47 \pm 0.53 \times 10^{-8}$	1 fold ↑	$4.93 \pm 2.33 \times 10^{-7}$	2 fold ↑
RN4220-M3	G2447T	WT	$1.45 \pm 1.18 \times 10^{-7}$	9 fold ↑	$1.59 \pm 0.77 \times 10^{-7}$	2 fold ↓
RN4220Δ*mutS*	**Control**	**Δ*mutS***	**$1.53 \pm 0.13 \times 10^{-7}$**	**WT**	**$1.06 \pm 0.21 \times 10^{-6}$**	**WT**
RN4220Δ*mutS*-M4	G2447T	*mutS* (revertant)	$1.50 \pm 0.76 \times 10^{-8}$	1 fold ↓ [E]	$1.63 \pm 0.37 \times 10^{-7}$	2 fold ↓
RN4220Δ*mutS*-M5	G2447T	*mutS* (revertant)	$1.69 \pm 1.41 \times 10^{-8}$	1 fold ↑ [E]	$1.29 \pm 0.68 \times 10^{-7}$	2 fold ↓
RN4220Δ*mutS* -M6	G2447T	*mutS* (revertant)	$8.39 \pm 4.85 \times 10^{-8}$	5 fold ↑ [E]	$7.37 \pm 4.26 \times 10^{-7}$	3 fold ↑
RN4220Δ*mutS* -M7	G2576T	*mutS* (revertant)	$5.63 \pm 2.07 \times 10^{-8}$	3 fold ↑ [E]	$1.42 \pm 0.71 \times 10^{-7}$	2 fold ↓
RN4220Δ*mutS*-M8	G2576T	*mutS* (revertant)	$1.44 \pm 0.72 \times 10^{-7}$	9 fold ↑ [E]	$2.27 \pm 1.37 \times 10^{-7}$	1 fold ↓
RN4220Δ*mutS*-M9	G2576T	Δ*mutS*	$2.78 \pm 1.55 \times 10^{-7}$	2 fold ↑	$4.98 \pm 4.51 \times 10^{-7}$	2 fold ↓
RN4220Δ*mutS*-M10	G2576T	Δ*mutS*	$3.17 \pm 1.18 \times 10^{-7}$	2 fold ↑	$1.70 \pm 0.88 \times 10^{-6}$	2 fold ↑
ST/03/2121	**Control**	**WT**	**$3.77 \pm 1.90 \times 10^{-8}$**	**WT**	**$5.04 \pm 3.29 \times 10^{-7}$**	**WT**
ST/03/2121-M11	T2504C	WT	$3.83 \pm 1.16 \times 10^{-8}$	1 fold ↑	$1.90 \pm 0.51 \times 10^{-7}$	3 fold ↓
ST/03/2121-M12	G2447T	WT	$5.33 \pm 1.17 \times 10^{-8}$	1 fold ↑	$3.19 \pm 1.15 \times 10^{-7}$	2 fold ↓

Cont.

Table 21. Summary of mutation frequencies of laboratory-selected mutants to rifampicin and fusidic acid (cont.).

Isolate	23S rRNA Mutation	Characteristics	Av. m freq to rif ± SD	Fold ↑ or ↓	Av. m freq to fus ± SD	Fold ↑ or ↓
ST/03/2121-M13	G2447T	WT	$2.48 \pm 1.39 \times 10^{-8}$	2 fold ↓	$1.43 \pm 0.71 \times 10^{-7}$	4 fold ↓
ST/03/2121-M14	G2447T	WT	$3.43 \pm 1.19 \times 10^{-8}$	1 fold ↓	$1.32 \pm 0.32 \times 10^{-7}$	4 fold ↓
ST/03/2121-M15	G2447T	WT	$1.02 \pm 0.39 \times 10^{-8}$	4 fold ↓	$8.18 \pm 2.98 \times 10^{-8}$	6 fold ↓
ST/03/2121-M16	Unknown mutation	WT	$5.94 \pm 2.06 \times 10^{-8}$	2 fold ↑	$1.17 \pm 0.88 \times 10^{-7}$	4 fold ↓
ST/03/2121-M17	Unknown mutation	WT	$3.06 \pm 1.23 \times 10^{-8}$	1 fold ↓	$1.26 \pm 0.18 \times 10^{-7}$	4 fold ↓

[A] Rifampicin (rif). [B] Standard deviation (SD). [C] Fold increase or decrease. [D] Fusidic acid (fus). [E] Revertants have been compared with RN4220.

Figure 29. Scatter and average of mutation frequencies of laboratory-selected linezolid-resistant mutants to rifampicin. Each dot represents the arithmetic mean mutation frequency for each mutant. The mean mutation frequency for a group of mutants of the same parentage is represented by a black line.

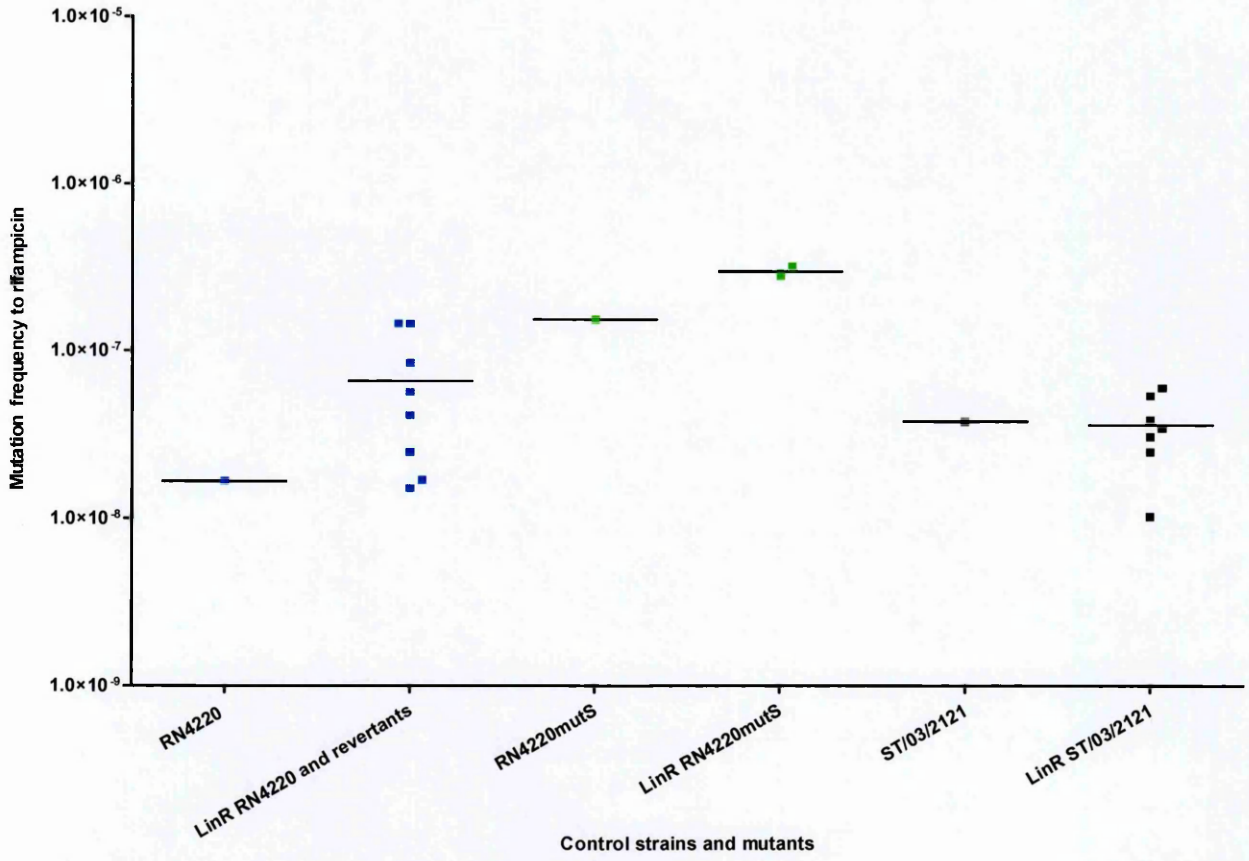
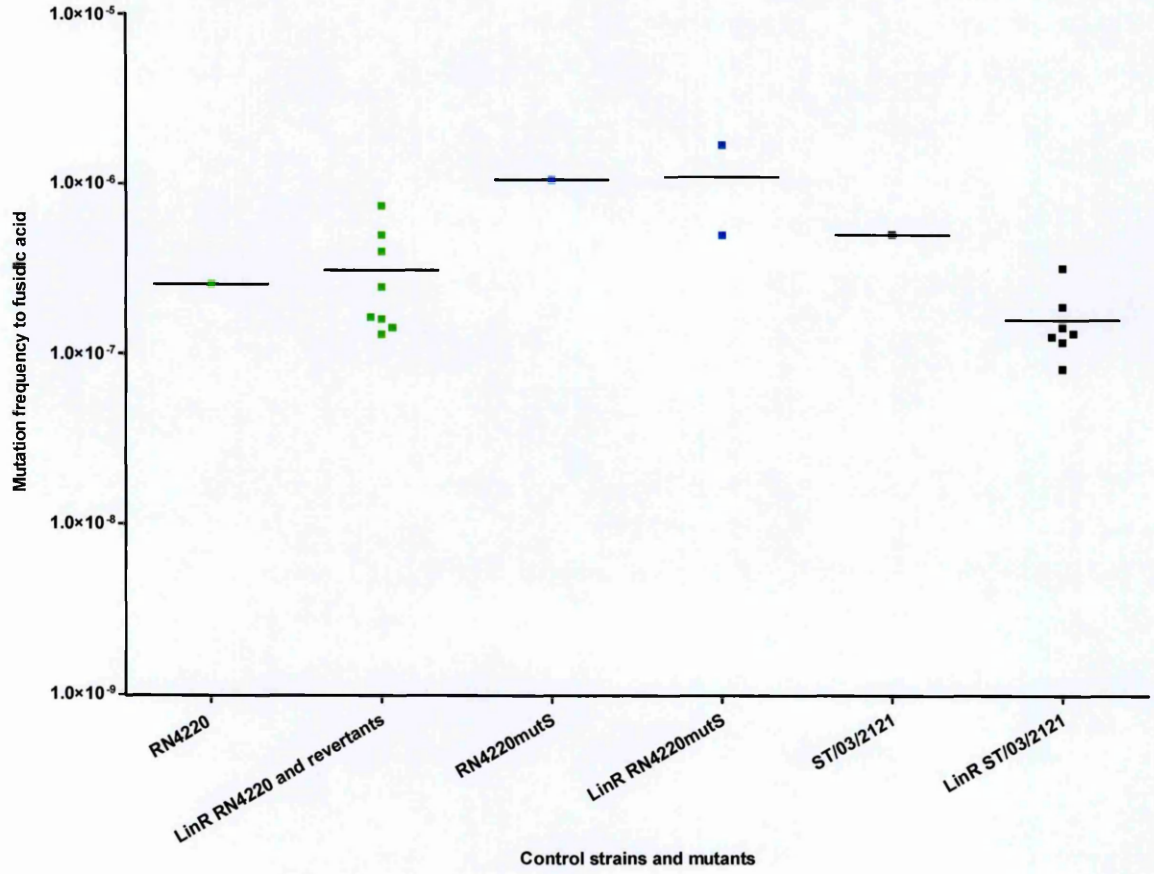


Figure 30. Scatter and average of mutation frequencies of laboratory-selected linezolid-resistant mutants to fusidic acid. Each dot represents the arithmetic mean mutation frequency for each mutant. The mean mutation frequency for a group of mutants of the same parentage is represented by a black line.



(M13, M14, M15, M17). Eight mutants had inconsistencies between rifampicin and fusidic acid, with both increases and decreases in mutation frequencies when compared with the parent strains. RN4220Δ*mutS* revertants produced mutation frequencies more comparable to RN4220 than to RN4220Δ*mutS*, consistent with accurate loss of the pGhost9^{+} plasmid and restoration of *mutS* function.

Mutant RN4220-M3 had a nine-fold increase in the average mutation frequency to rifampicin. Looking at the individual mutation frequencies, one experiment produced a mutation frequency substantially higher than the other two experiments, resulting in an elevated average mutation frequency and a large increase when compared with the parent strain, possibly due to a jackpot culture. A mutation can occur at any time during the period of growth of a culture and therefore, the number of mutants at the end of the experiment represents the number of independent mutation events as well as the accumulation of clones of the mutants themselves. Therefore, a mutation occurring relatively early during the period of growth, known as a "jackpot culture", will result in a greater final mutation frequency (Saunders *et al.*, 2003). This increase in mutation frequency to rifampicin was not reflected in the mutation frequency to fusidic acid. Mutant RN4220Δ*mutS*-M8 had a nine-fold increase in rifampicin mutation frequency when compared with the parent strain, again without any parallel increase in the mutation frequency to fusidic acid.

These results show that there is little evidence that selection for linezolid resistance could co-select for hypermutability. Increases in the mutation frequencies to rifampicin occurred in a number of mutants, but this was not necessarily echoed in the mutation frequencies to fusidic acid; only three mutants had increases in mutation frequencies to both rifampicin and fusidic acid. These increases ranged between two- and five-fold. The type of mutation conferring resistance to linezolid did not seem to affect the mutation frequency of the mutant.

3.2.6 Fosfomycin disc tests to detect hypermutability

This method was used as a screen for hypermutators (see section 2.9.2). Briefly, bacteria were suspended to a density of a 1.0 McFarland standard and swabbed over an ISO agar plate before a 50 μg disc was applied. After 24 hours incubation, the numbers of colonies in the zone of clearing

were counted and the zone diameter measured. The mean of three replicates was calculated and the results plotted. RN4220Δ*mutS* produced a mean of 292 colonies in the zone of clearing, five times that of RN4220, which produced a mean of 60 (Table 22 and Figure 31). ST/03/2121 produced a mean of 251, four times that of RN4220. ST/03/2122 produced nearly three times as many colonies in the zone of clearing as RN4220. The number of colonies produced in the zone of clearing decreased for 14 of 17 linezolid-resistant mutants. Mutants gave between 14 and 174 colonies fewer in the zone of clearing than parent strains, with mutants of ST/03/2121 producing the largest drops in colony numbers. Only two mutants produced more colonies in the zone of clearing than their parent strains (M5, M7) and these only produced, on average, 12 to 19 more mutants than their parent, RN4220. Mutant M4 produced the same number of colonies in the zone as its parent strain. The mutants which had increased mutation frequencies to rifampicin and fusidic acid (M1, M2, M6, M10) (see section 3.2.5) did not produce more colonies in the zone of clearing than their parent strains.

All mutants had increased zone diameters compared with their parent strains (Table 22 and Figure 32). Zone diameters increased considerably for linezolid-resistant mutants of RN4220 and RN4220*mutS*, however increases in zone sizes of mutants of ST/03/2121 were less marked. Zone diameters increased by 0.2 mm to 12.5 mm. Fourteen mutants had decreases in the number of colonies produced in the zone of clearing and increases in zone diameter compared with their parent strains. One of these, mutant M17, produced, on average, 146 colonies fewer and a diameter 12.5 mm larger than its parent strain, ST/03/2121. The remaining three had increases in the number of mutants produced in the zone of clearing and in the zone diameter compared with their parent strains. The zone diameters correlated well with fosfomycin MICs (by agar dilution), with increased zone sizes relating to decreased MICs. There was a difference in both MICs and zone sizes between parents and mutants, possibly indicating some loss of mutability. Approximately a 5 mm change in zone size was required to see a change in MIC. Overall, there was no indication that there is co-selection of hypermutability with linezolid resistance using fosfomycin as an indicator, in fact there seems to be some evidence pointing towards a reduction in mutability.

Table 22. Number of colonies in the zone of inhibition and zone diameters surrounding fosfomycin discs (50 μg) for laboratory-selected linezolid-resistant mutants.

Strain	Mutation	Characteristics	Lin MIC (mg/L)	No. of colonies in zone in each of 3 experiments			Average ± SD	Zone diameter (mm) in each of 3 experiments			Average ± SD
RN4220	**N/A**	**WT**	**2**	**66**	**53**	**59**	**60 ± 7**	**41.8**	**42.0**	**43.7**	**42.5 ± 1**
RN4220-M1	G2447T	WT	32	30	57	50	**46 ± 14**	51.6	50.4	50.4	**50.8 ± 1**
RN4220-M2	G2447T	WT	2	43	37	53	**44 ± 8**	48.3	50.3	48.4	**49.0 ± 1**
RN4220-M3	G2447T	WT	32	35	31	17	**28 ± 9**	52.5	53.7	51.8	**52.7 ± 1**
RN4220Δ*mutS*	**N/A**	**WT**	**32**	**302**	**267**	**306**	**292 ± 21**	**43.8**	**43.8**	**44.3**	**43.9 ± 0**
RN4220Δ*mutS*-M4	G2447T	*mutS* (revertant)	16	59	53	67	**60 ± 7**	50.5	50.9	51.5	**51.0 ± 1**
RN4220Δ*mutS*-M5	G2447T	*mutS* (revertant)	32	73	58	84	**72 ± 13**	52.0	53.1	54.2	**53.1 ± 1**
RN4220Δ*mutS*-M6	G2447T	*mutS* (revertant)	32	30	35	34	**33 ± 3**	49.5	48.8	47.2	**48.5 ± 1**
RN4220Δ*mutS*-M7	G2576T	*mutS* (revertant)	32	96	91	50	**79 ± 25**	46.1	45.0	46.6	**45.9 ± 1**
RN4220Δ*mutS*-M8	G2576T	*mutS* (revertant)	32	30	38	46	**38 ± 8**	44.9	45.8	47.1	**45.9 ± 1**
RN4220Δ*mutS*-M9	G2576T	::*mutS*	16	241	212	269	**241 ± 29**	47.0	49.3	46.8	**47.7 ± 1**
RN4220Δ*mutS*-M10	G2576T	::*mutS*	4	146	182	150	**159 ± 20**	45.0	44.9	45.3	**45.1 ± 0**
ST/03/2121	**N/A**	**WT**	**8**	**276**	**243**	**234**	**251 ± 22**	**42.4**	**41.3**	**42.4**	**42.0 ± 1**
ST/03/2121-M11	T2504C	WT	64	70	71	89	**77 ± 11**	43.6	41.4	42.7	**42.6 ± 1**
ST/03/2121-M12	G2447T	WT	16	146	163	171	**160 ± 13**	42.1	44.3	40.2	**42.2 ± 2**

Cont.

Table 22. Number of colonies in the zone of inhibition and zone diameters surrounding fosfomycin discs for laboratory-selected linezolid-resistant mutants (cont.).

Strain	Mutation	Characteristics	Lin MIC (mg/L)	No. of colonies in zone in each of 3 experiments			Average ± SD	Zone diameter (mm) in each of 3 experiments			Average
ST/03/2121-M13	G2447T	WT	16	86	120	125	110 ± 21	43.0	44.0	44.4	43.8 ± 1
ST/03/2121-M14	G2447T	WT	16	102	122	109	111 ± 10	44.7	42.6	43.8	43.7 ± 1
ST/03/2121-M15	G2447T	WT	32	127	53	106	95 ± 38	42.9	43.4	42.4	42.9 ± 1
ST/03/2121-M16	Unknown	WT	8	118	157	127	134 ± 20	45.6	44.4	42.5	44.2 ± 2
ST/03/2121-M17	Unknown	WT	32	112	97	105	105 ± 8	52.2	55.3	56.1	54.5 ± 2
ST/03/2122	**N/A**	**WT**	**1**	**128**	**148**	**183**	**153 ± 28**	**39.3**	**40.2**	**39.4**	**39.6 ± 0**

Figure 31. Number of colonies in the zone of clearing surrounding fosfomycin discs for laboratory-selected linezolid-resistant mutants. Standard deviations are represented by error bars. RN4220 is designated RN, RN4220Δ*mutS* is designated mutS and linezolid-resistant mutants have a -M prefix and follow after their parent strain.

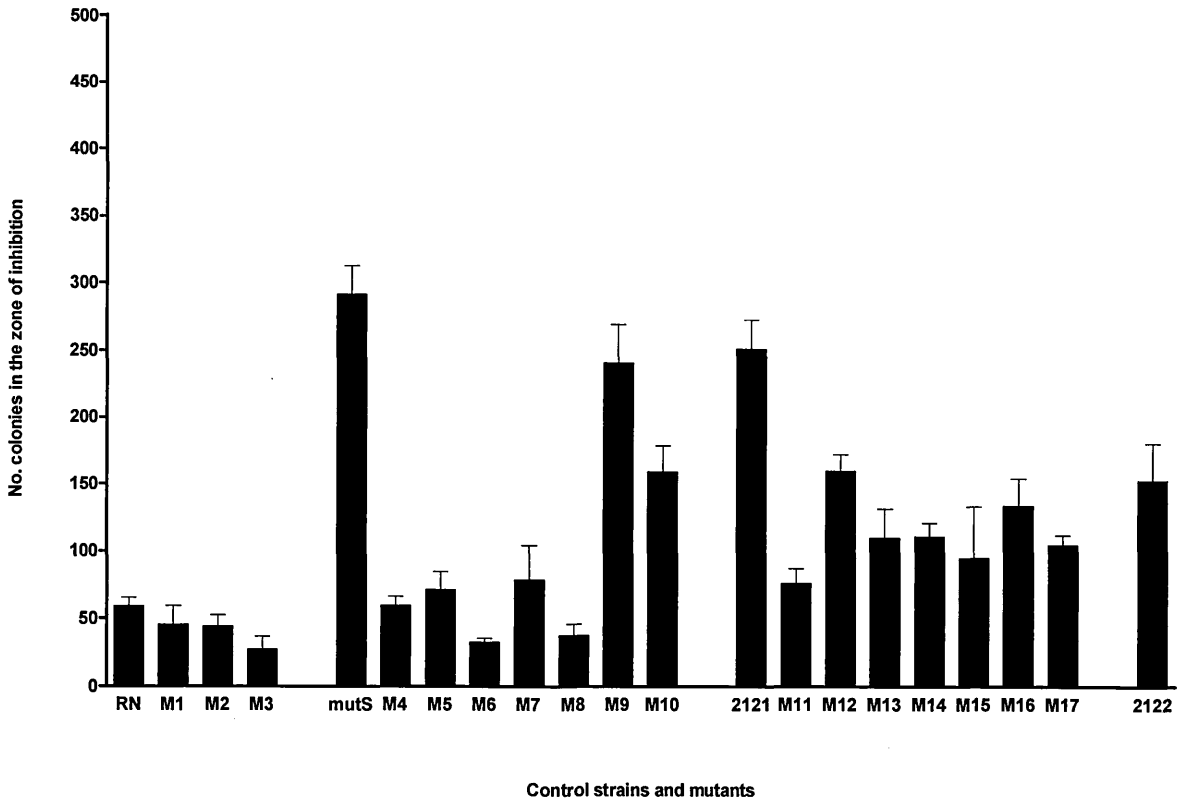
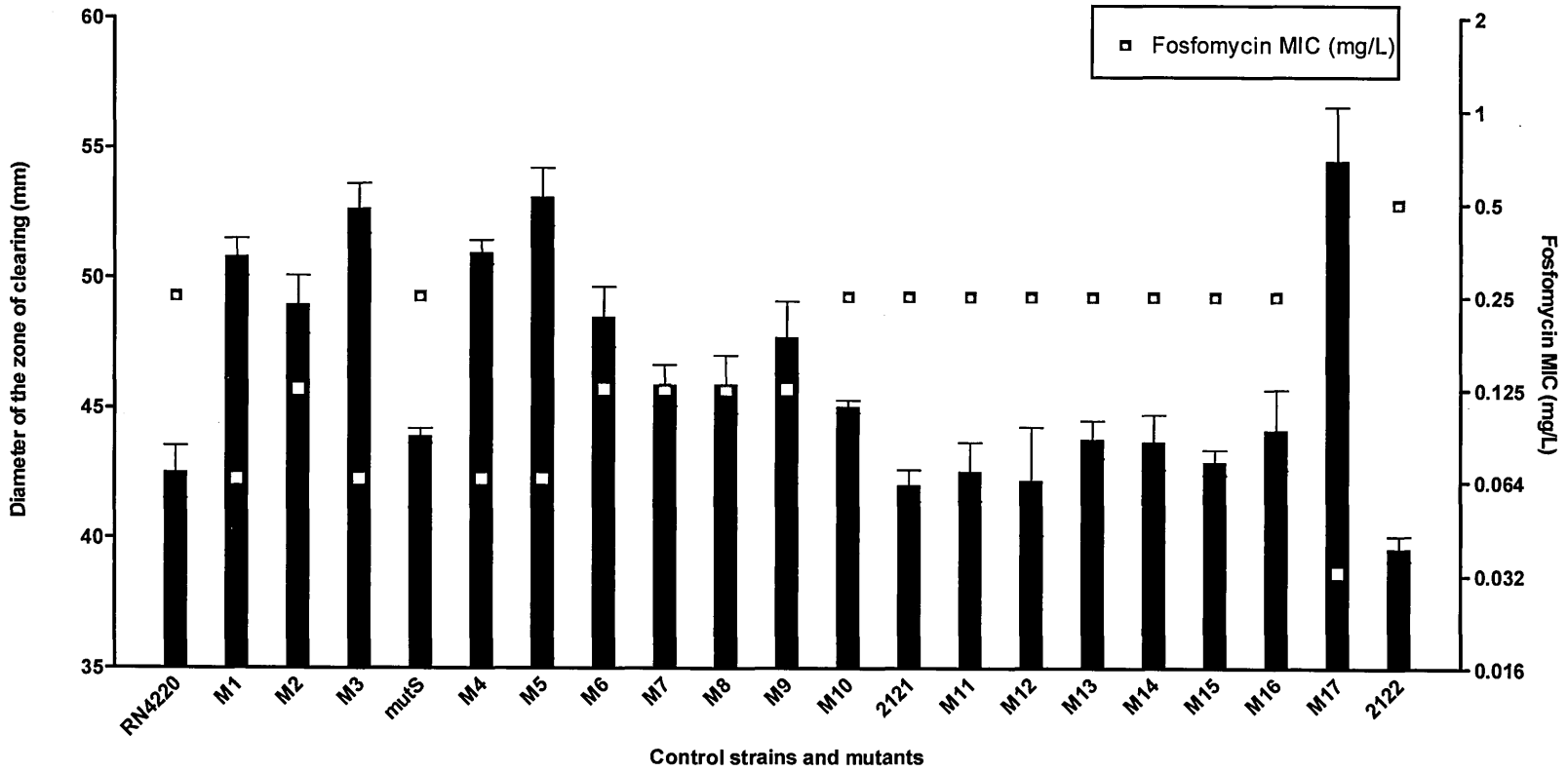


Figure 32. Diameter of the zone of inhibition surrounding fosfomycin discs (50 μg) and fosfomycin MICs produced by laboratory-selected linezolid-resistant mutants. Standard deviations are represented by error bars. RN4220Δ*mutS* is designated mutS, linezolid-resistant mutants have a -M prefix and follow after their parent strains.



3.2.7 Summary

Many laboratory-selected linezolid-resistant mutants proved unstable. However, 17 linezolid-resistant mutants were selected. These mutants had a variety of mutations (G2576T, G2447T, T2504C), as detected by PCR-RFLP, DNA sequencing and pyrosequencing. Pyrosequencing and hybridization revealed that 23S rRNA gene copy number varied between parents and their linezolid-resistant mutants. Chloramphenicol MICs increased for some of the mutants. Some RN4220Δ*mutS*-derived mutants reverted to wild-type, with the excision of the pGhost9^{+} plasmid, carrying an *erm* determinant, inserted to disrupt the *mutS* gene. The evidence to support the co-selection of hypermutability and linezolid resistance is lacking, with no linezolid-resistant mutants producing increased numbers of mutants to all three antibiotics tested.

3.3 Loss of erythromycin resistance with the emergence of linezolid resistance

Whilst raising linezolid-resistant mutants *in vitro*, it was noted that some mutants of RN4220Δ*mutS* lost the pGhost9^{+} plasmid, inserted to disrupt the *mutS* gene. This plasmid contained an *erm*(B) resistance marker. The next section investigates this further.

3.3.1 Characterization of mutants

A group of erythromycin-resistant, with *erm*(B) and *erm*(C) genes, and erythromycin-susceptible clinical isolates were grown in the presence and absence of erythromycin along with increasing concentrations of linezolid to try to establish, firstly, whether erythromycin would delay the emergence of linezolid resistance in erythromycin-resistant isolates, and secondly, whether reversion to erythromycin susceptibility consistently occurred with the emergence of linezolid resistance (Table 23) (see section 2.5.2). The erythromycin-resistant strains were tested for *erm* determinants by PCR and their inducibility was addressed by double disc tests (Table 23) (see section 2.3.5)

Table 23. Characteristics of strains used in the selection of linezolid resistance.

Strain	PFGE type	*erm* determinant	Ery MIC (mg/L)	Constitutive or inducible	Linezolid MIC (mg/L)
RN4220	N/A	Not present	1	N/A	4
RN4220Δ*mutS*	N/A	*erm*(B)	>256	Constitutive	4
7499	EMRSA-15 B1	Not present	≤0.25	N/A	4
7500	EMRSA-15 B1	*erm*(C)	>256	Inducible	4
7717	EMRSA-15 B3	Not present	≤0.25	N/A	4
7501	EMRSA-15 B3	*erm*(C)	>256	Inducible	4

Strains were passaged on agar containing a constant concentration of linezolid until sufficient growth had occurred, before then being passaged at the next concentration. Upon being able to grow on agar containing 10 mg/L of linezolid, mutants were passaged five times at this concentration to try to stabilize resistance. The number of days each strain spent plated on agar containing each concentration of linezolid was recorded. The experiment was repeated four times using 100 mg/L of erythromycin (Appendix B Table 55, Figure 64, Figure 65, Figure 66, Figure 67). The fifth experiment was conducted in the presence of 8 mg/L of erythromycin, a concentration around the serum peak (the maximum concentration in the body after dosing ranges from <1-14 mg/L) (Figure 36) (Bryskier and Butzler, 1997).

Mutants selected from the five experiments underwent linezolid and erythromycin MIC determinations (see section 2.3.2). Mutants were screened for G2576T, T2504C and G2505A mutations by RFLP of a 694 bp PCR product of the 23S rRNA genes with the restriction endonucleases *Nhe*I, *Hin*1I and *Eco*RV, respectively (see section 2.7.4). Sequencing and pyrosequencing confirmed the presence or absence of the above mutations as well as T2500A, A2503G, G2445T and G2447T mutations (see section 2.7). Initially, erythromycin MIC determinations confirmed the presence or absence of *erm* determinants in the mutants and was later confirmed by PCR (Figure 33 [A] and [B]) (see section 2.7.2). Mutants underwent PFGE to confirm parentage (Figure 34) (see section 2.6.3).

Forty-two mutants were selected from five repeated experiments (Table 24). Mutants were numbered M1 to M42. Linezolid MICs ranged from 4 to 64 mg/L. Twenty-six mutants were resistant to linezolid, the remaining 16 were susceptible. Of the 16 mutants that were linezolid-susceptible, ten were grown in the presence of erythromycin and linezolid. Linezolid MICs ranged from 4 to 64 mg/L for mutants grown in the absence of erythromycin and from 4 to 8 mg/L for mutants grown in the presence of erythromycin. Linezolid resistance was unstable, with some mutants able to grow on agar containing 10 mg/L linezolid yet, after one passage on antibiotic-free agar, with a MIC of 4 mg/L.

Eleven mutants had G2576T mutations, three had G2447T mutations, six had T2504C mutations and six isolates had unidentifiable mechanisms of resistance (all six with linezolid MICs of 8 mg/L). Four out of 14 mutants, selected in the presence of erythromycin, were resistant to linezolid

Figure 33. PCR to detect the presence of an *erm* determinant in linezolid-resistant mutants. **(A)** *erm*(B) determinant. Lanes 1 and 6, 123 bp ladder, lane 2 RN4220Δ*mutS* control, lane 3 M35, lane 4 M36, lane 5 water control. **(B)** *erm*(C) determinant. Lanes 1 and 9, 123 bp ladder, lane 2 M39, lane 3 M38, lane 4 M41, lane 5 M42, lane 6 7500 control, lane 7 7501 control, lane 8 water control.

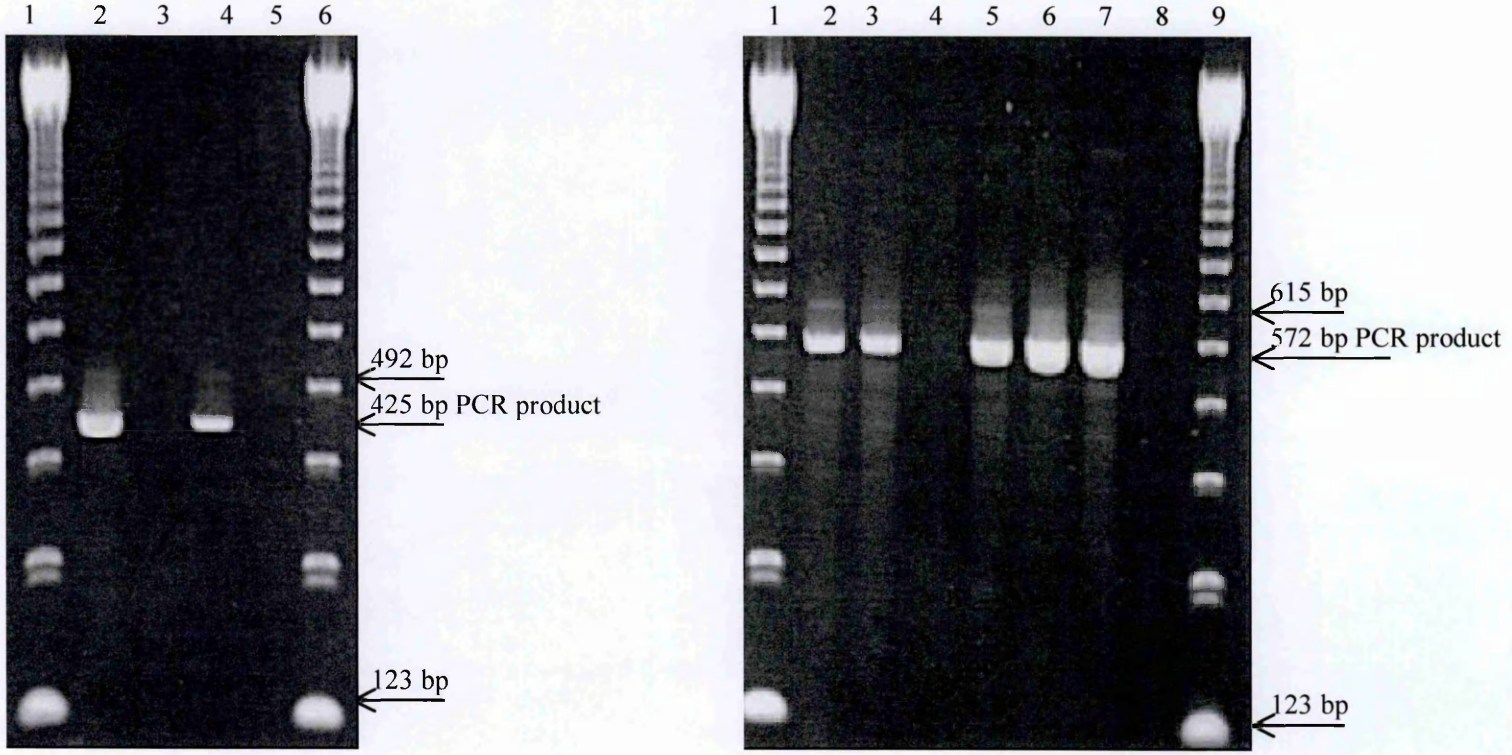


Figure 34. PFGE of linezolid-resistant mutants to verify parentage. Lanes 1 and 16 lambda ladder, lane 2 7717 control, lane 3-6 M22-M25, lane 7 7501 control, lane 8-15 M26-M33.

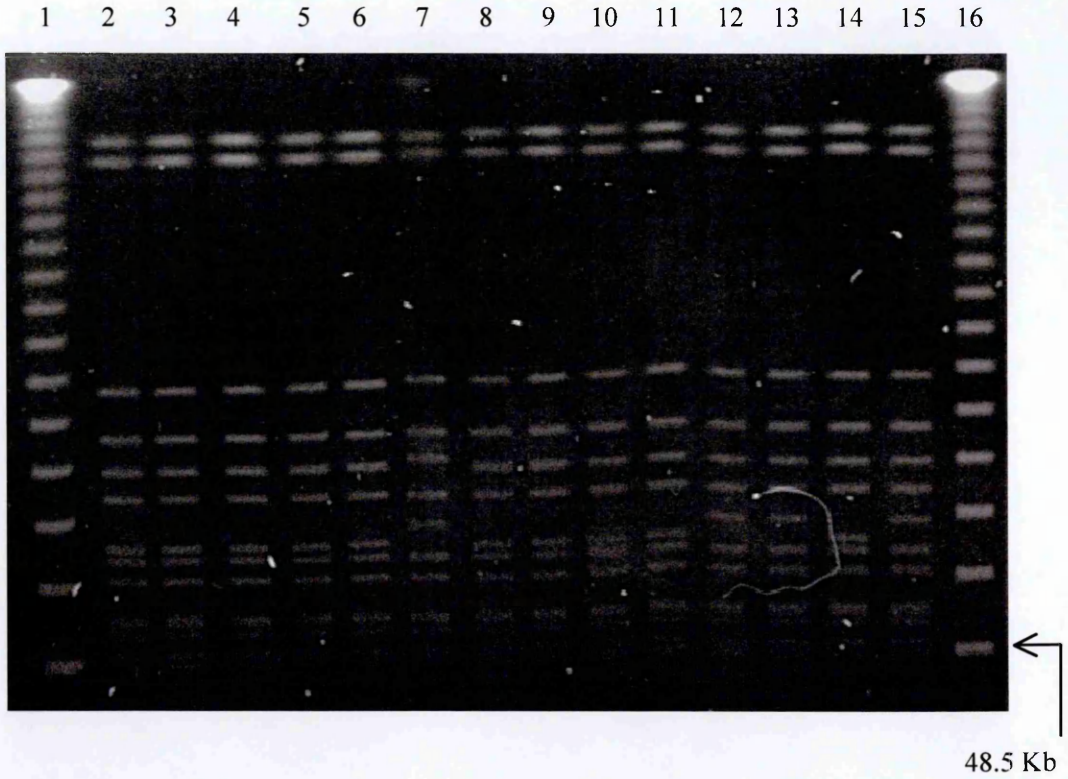


Table 24. Linezolid and erythromycin MICs and resistance mechanisms of mutants selected in the five replicated experiments.

Mutant	Grown on	Ery[A] MIC (mg/L)	Lin MIC (mg/L)	Loss of *erm*	Mutation
RN4220	**N/A**	**1**	**4**	**N/A**	**N/A**
M1	Lin	0.25	16	N/A	G2576T
M2	Lin	0.25	64	N/A	G2576T
M3	Lin	0.5	32	N/A	G2576T
M34	Lin	0.25	16	N/A	G2447T
RN4220Δ*mutS*	**N/A**	**>256**	**4**	**N/A**	**N/A**
M4	Lin	>256	32	N	G2576T
M5	Lin	>256	64	N	G2576T
M6	Lin	>256	16	N	G2576T
M35	Lin	0.50	16	Y	G2447T
M7 [B]	Lin and Ery	>256	4	N	T2504C
M8 [B]	Lin and Ery	>256	8	N	T2504C
M9 [B]	Lin and Ery	>256	8	N	G2576T
M36 [C]	Lin and Ery	>256	4	N	N/A
7499	**N/A**	**0.25**	**4**	**N/A**	**N/A**
M10	Lin	0.25	32	N/A	T2504C
M11	Lin	0.25	64	N/A	T2504C

Cont.

Table 24. Linezolid and erythromycin MICs and resistance mechanisms of mutants selected in the five replicated experiments (cont.).

Mutant	Grown on	Ery MIC (mg/L)	Lin MIC (mg/L)	Loss of *erm*	Mutation
M12	Lin	0.25	32	N/A	T2504C
M13	Lin	0.25	64	N/A	T2504C
M37	Lin	0.25	4	N/A	N/A
7500	**N/A**	**>256**	**4**	**N/A**	**N/A**
M14	Lin	0.50	4	Y	N/A
M15	Lin	0.125	8	Y	Unknown mutation
M16	Lin	>256	64	N	G2447T
M17	Lin	>256	32	N	G2576T
M38	Lin	>256	32	N	G2576T
M18 [B]	Lin and Ery	>256	4	N	N/A
M19 [B]	Lin and Ery	>256	8	N	Unknown mutation
M20 [B]	Lin and Ery	>256	4	N	N/A
M21 [B]	Lin and Ery	>256	4	N	N/A
M39 [C]	Lin and Ery	>256	8	N	Unknown mutation
7717	**N/A**	**0.25**	**4**	**N/A**	**N/A**
M22	Lin	0.25	8	N/A	Unknown mutation
M23	Lin	0.125	64	N/A	G2576T
M24	Lin	0.125	8	N/A	Unknown mutation

Cont.

Table 24. Linezolid and erythromycin MICs and resistance mechanisms of mutants selected in the five replicated experiments (cont.).

Mutant	Grown on	Ery MIC (mg/L)	Lin MIC (mg/L)	Loss of *erm*	Mutation
M25	Lin	0.25	8	N/A	Unknown mutation
M40	Lin	0.25	32	N/A	G2576T
7501	**N/A**	**>256**	**4**	**N/A**	**N/A**
M26	Lin	>256	4	Y	N/A
M27	Lin	>256	4	N	N/A
M28	Lin	>256	4	Y	N/A
M29	Lin	0.25	4	Y	N/A
M41	Lin	>256	4	N	N/A
M30 [B]	Lin and Ery	>256	4	N	N/A
M31 [B]	Lin and Ery	>256	4	N	N/A
M32 [B]	Lin and Ery	>256	4	N	N/A
M33 [B]	Lin and Ery	>256	4	N	N/A
M42 [C]	Lin and Ery	>256	4	N	N/A

[A] Erythromycin (Ery)

[B] Variants grown in the presence of increasing concentrations of linezolid and a constant 100 mg/L of erythromycin.

[C] Variants grown in the presence of increasing concentrations of linezolid and a constant 8 mg/L of erythromycin.

(MICs 8 mg/L); two with T2504C mutations and one with a G2576T mutation were identified, one mutant had no identifiable mechanism of resistance. Mutations G2576T, G2447T and T2504C were all identified in linezolid-resistant isolates selected in the absence of erythromycin.

Six of 14 erythromycin-resistant mutants, grown in the absence of erythromycin, reverted to erythromycin susceptibility with the loss of an *erm* determinant; one of four *erm*(B) mutants and five of ten *erm*(C) mutants. Of these six, only one had a detectable mutation (G2447T). Four of the six had linezolid MICs of 4 mg/L. No erythromycin-resistant mutants grown in the presence of erythromycin lost an *erm* determinant.

The average time taken (experiments one to four, with 100 mg/L erythromycin) for linezolid resistance to emerge (i.e., for mutants to develop the ability to grow with 6 mg/L of linezolid) ranged from 11 to 44 days, with resistance developing quickest in the hypermutable isolate RN4220Δ*mutS* (Table 25 and Figure 35 and Appendix B Figure 64, Figure 65, Figure 66 and Figure 67). Erythromycin-susceptible strains took between 23 and 37 days to grow in the presence of 6 mg/L linezolid. Erythromycin-resistant strains took between 11 and 44 days to grow in the presence of 6 mg/L linezolid. Growing erythromycin-resistant isolates in the presence of erythromycin and increasing concentrations of linezolid delayed the emergence of linezolid resistance, on average, by 17 days in mutants of strain 7500 and 19 days in mutants of strain 7501. Growing the hypermutable strain in the presence of erythromycin did not delay the time taken for linezolid resistance to emerge. Mutants took 13 to 54 days to grow in the presence of 6 mg/L linezolid with the erythromycin concentration at 8 mg/L (Table 25 and Figure 36 and Appendix B Table 52). Erythromycin-susceptible strains took between 29 and 54 days to grow in the presence of 6 mg/L linezolid. Erythromycin-resistant strains took between 13 and 46 days to grow in the absence of erythromycin and in the presence of 6 mg/L linezolid. Linezolid resistance was delayed by eight and 23 days in erythromycin-resistant strains 7500 and 7501, respectively, by growing them in the presence of 8 mg/L erythromycin. Again, linezolid resistance emerged quickest in the hypermutable strain, and here, the addition of erythromycin made no difference in the time taken for linezolid resistance to emerge. It resulted in an increase of four days in strain 7501 and a decrease of nine days in strain 7500. However, this experiment (with the addition of 8 mg/L

Table 25. Summary of time taken for mutants to grow on agar containing 6 mg/L of linezolid. (See Appendix B Table 52 for more detailed results).

			Average time taken (days)	
Parent strain	**Erythromycin R/S**	**Grown in the presence of**	**Experiments 1-4**	**Experiment 5**
RN4220	S	Linezolid	24	29
RN4220Δ*mutS*	R	Linezolid	11	13
RN4220Δ*mutS*	R	Linezolid and erythromycin	11	13
7499	S	Linezolid	37	34
7500	R	Linezolid	26	38
7500	R	Linezolid and erythromycin	43	46
7717	S	Linezolid	23	54
7501	R	Linezolid	25	23
7501	R	Linezolid and erythromycin	44	46

Figure 35. Summary of the average time taken for linezolid resistance to emerge in each of the strains over the course of five replicated experiments (with (EL) and without (L) erythromycin (100 mg/L)). Each shaded area represents the number of days each concentration of linezolid had to be maintained before it could be driven higher. (See Appendix B Figure 64, Figure 65, Figure 66 and Figure 67 for individual experiments).

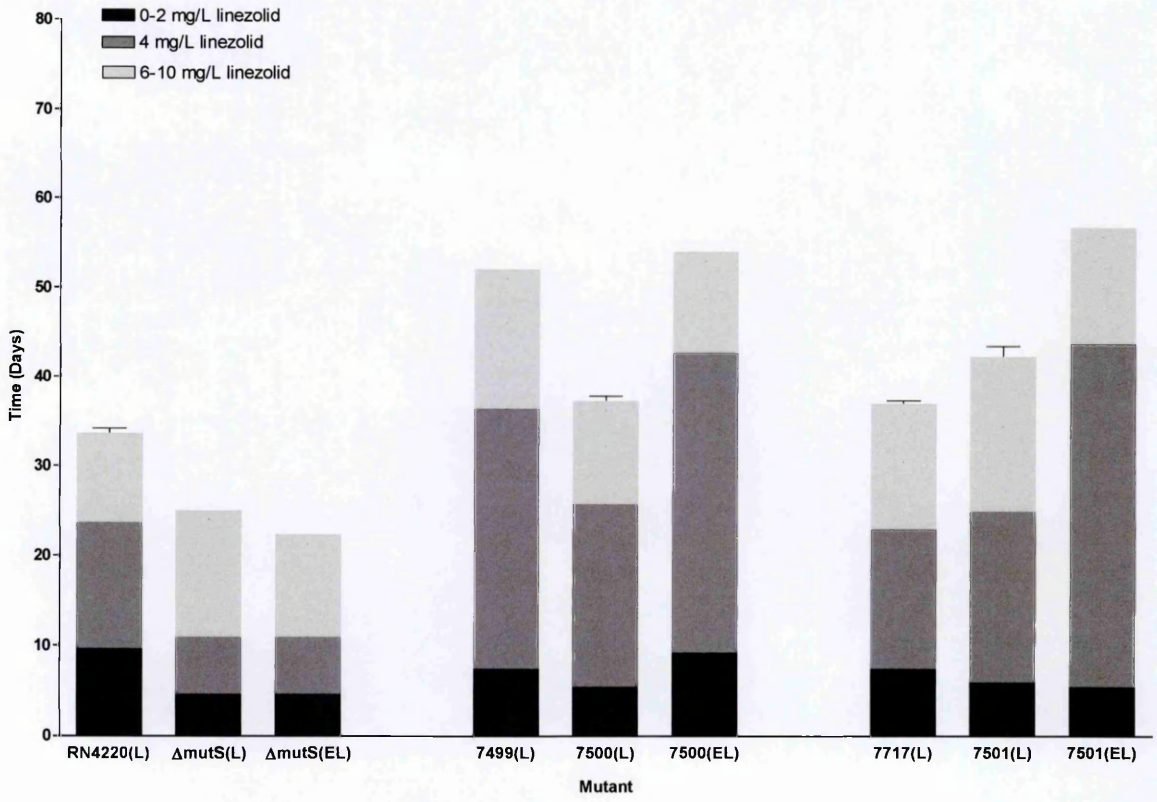
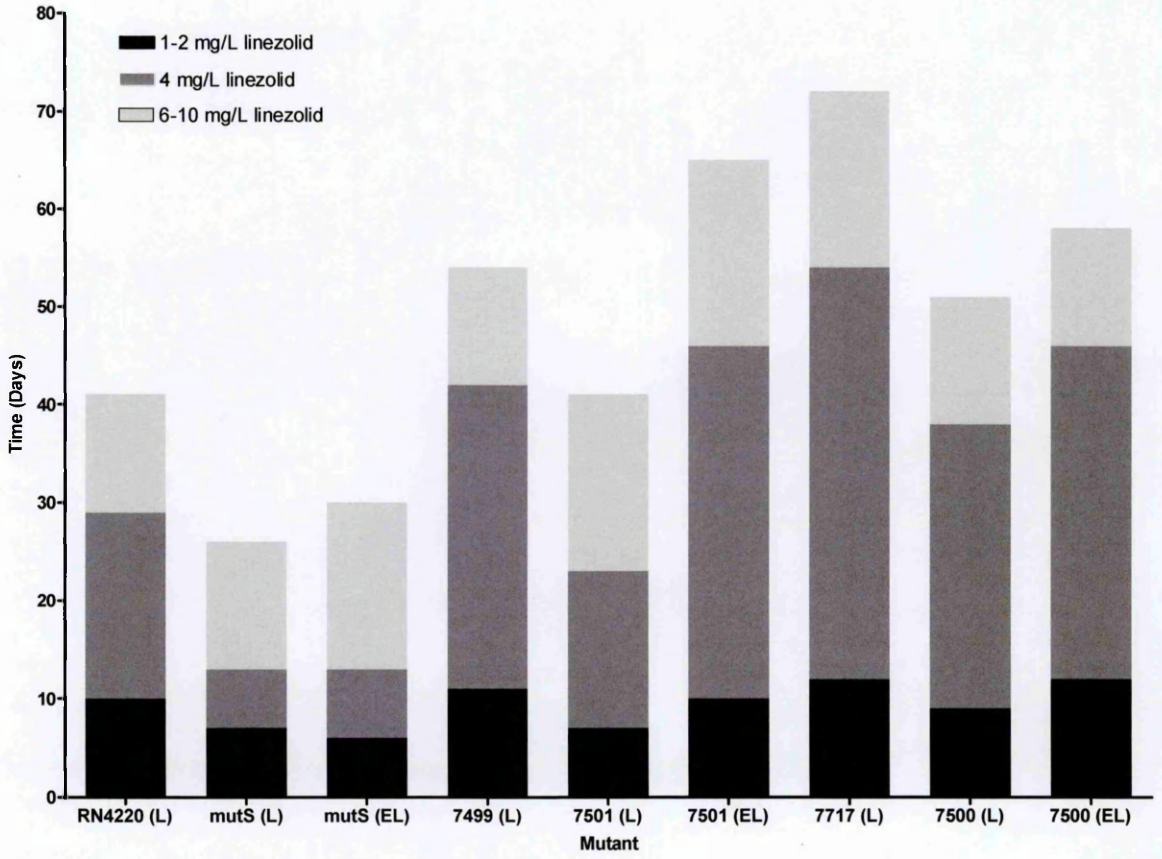


Figure 36. Summary of the average time taken for linezolid resistance to emerge in each of the strains (with (EL) and without (L) erythromycin (8 mg/L)).

Each shaded area represents the number of days each concentration of linezolid had to be maintained before it could be driven higher.



erythromycin) was only carried out once and the four experiments using 100 mg/L of erythromycin show variation between them.

To establish whether the growth of erythromycin-resistant strains was hindered in the presence of erythromycin, growth curves of erythromycin-resistant strains were carried out in the presence and absence of 100 mg/L of erythromycin (Figure 37) (see section 2.4.3). The results showed no difference in growth in the presence or absence of erythromycin. Additionally, the stability of linezolid in agar over a period of five days was investigated and results showed no evidence for the degradation of linezolid (see section 2.5.2.1).

Hybridization and pyrosequencing were used in conjunction to determine the number of mutated 23S rRNA gene copies in linezolid-resistant mutants and parents (see section 2.7.7 and 2.8). Hybridization revealed 24 of 42 mutants had altered 23S rRNA gene copy number compared with their parent strains, the remaining 18 had the same copy number as their parent strains (Table 26 and Figure 38). Of the 24 mutants with changed copy number, 21 had increased, three had decreased. All three with a decreased copy number, when compared with their parent strains, had decreased by one. Of the 21 mutants with an increased copy number when compared with their parent strains, 14 had increased by one copy, four mutants had increased by two copies, one had increased by three copies and two had increased by four copies. Pyrosequencing and hybridization combined, revealed mutants with a G2576T mutation had 15-65% of copies mutated, which equated to between one and four mutated 23S rRNA gene copies (Table 26). Mutants with a T2504C mutation had 33-48% of copies mutated, which equated to two or three mutated 23S rRNA gene copies. One mutant with a A2503G mutation had 14% of copies mutated, which equated to one mutated 23S rRNA gene copy.

Figure 37. Growth curves of erythromycin-resistant strains and isolates in the presence and absence of erythromycin (Ery) (100 mg/L).

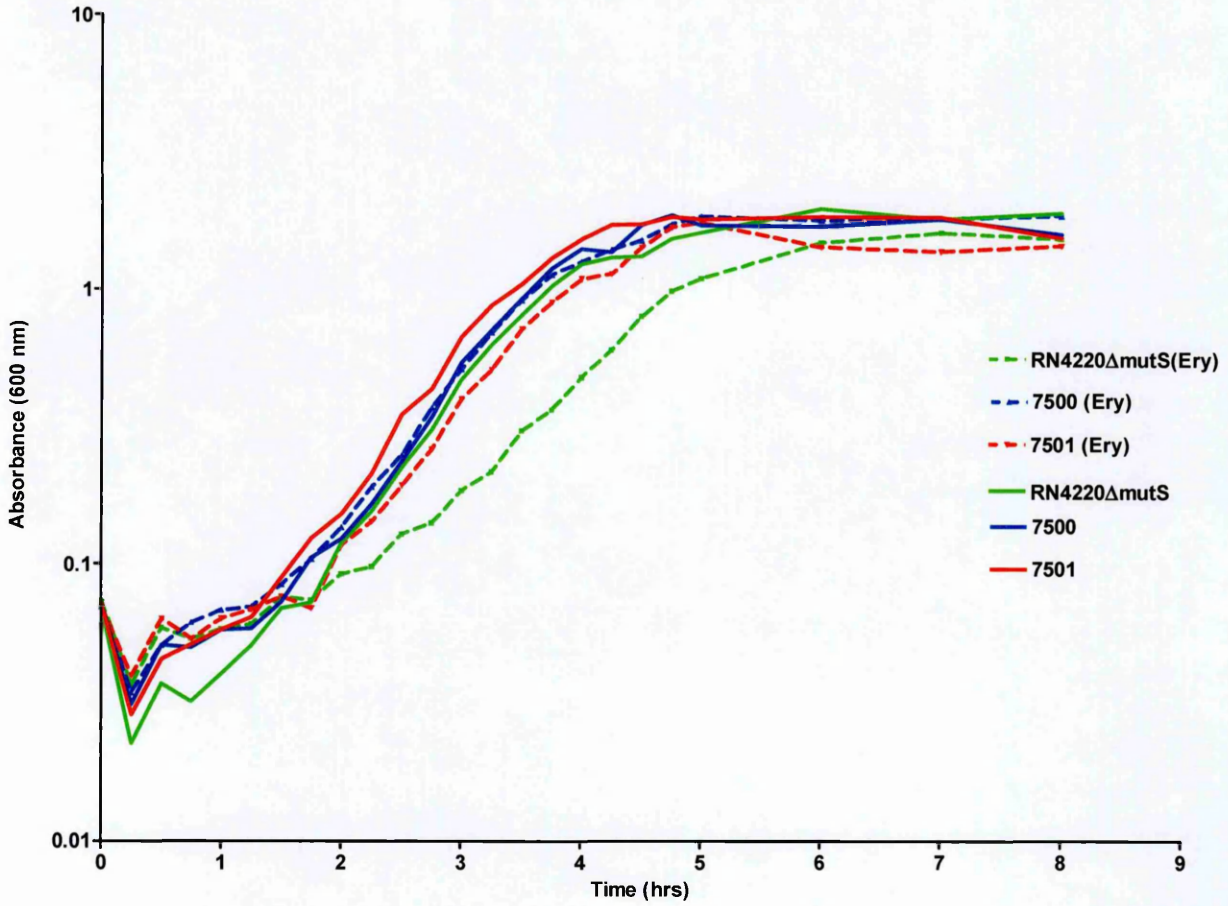


Table 26. Pyrosequencing and hybridization results of all mutants selected during the course of five replicated experiments. Numbers in brackets refer to increase or decrease in 23S rRNA when compared with parent strains. (See Appendix B Table 55 for a complete summary of pyrosequencing and hybridization results).

Strain	Mutation	Lin MIC (mg/L)	23S rRNA gene copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WT copies
RN4220	**WT**	**4**	**6**	**WT**	**WT**	**WT**
M1	G2576T	16	6	51.5	48.5	3:3
M2	G2576T	64	6	64.8	35.2	4:2
M3	G2576T	16	6	46.6	53.4	3:3
M34	G2447T	16	6	N/A	N/A	N/A
RN4220Δ*mutS*	**WT**	**4**	**6**	**WT**	**WT**	**WT**
M4	G2576T	16	8 (+2)	46.0	54.0	4:4
M5	G2576T	32	6	54.7	45.3	3:3
M6	G2576T	16	5 (-1)	41.2	58.8	2:3
M35	G2447T	16	6	N/A	N/A	N/A
M7	T2504C	8	6	36.8	63.2	2:4
M8	T2504C	8	6	12.5	87.5	1:5
M9	G2576T	8	5 (-1)	37.2	62.8	2:3
M36	N/A	4	6	N/A	N/A	N/A

Cont.

Table 26. Pyrosequencing and hybridization results of all mutants selected during the course of five replicated experiments (cont.).

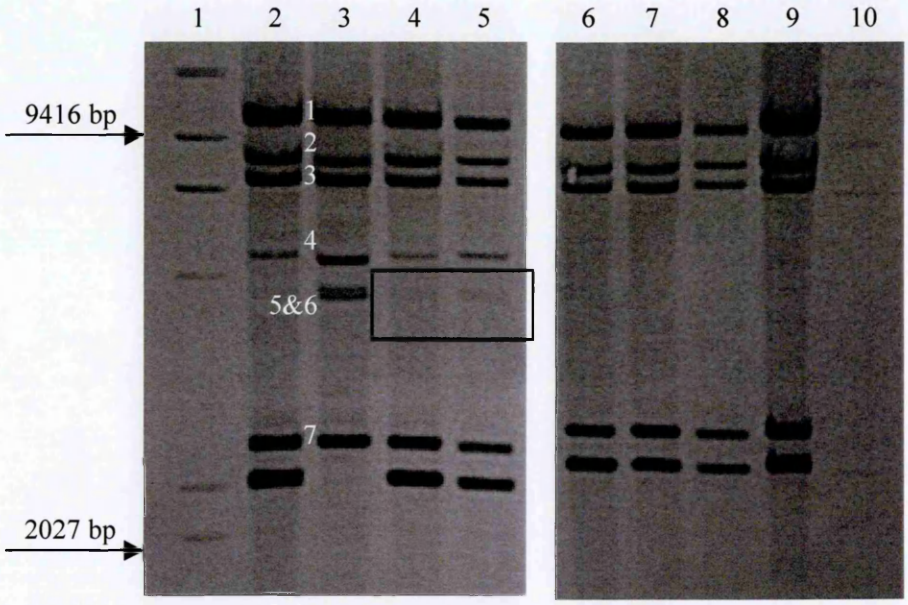
Strain	Mutation	Lin MIC (mg/L)	23S rRNA gene copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WT copies
7499	**WT**	**4**	**5**	**WT**	**WT**	**WT**
M10	T2504C	16	6 (+1)	48.3	51.7	3:3
M11	T2504C	16	6 (+1)	32.9	67.5	2:4
M12	T2504C	16	7 (+2)	42.3	57.7	3:4
M13	T2504C	16	7 (+2)	30.0	70.0	2:5
M37	N/A	4	5	N/A	N/A	N/A
7500	**WT**	**4**	**5**	**WT**	**WT**	**WT**
M14	N/A	4	8 (+3)	N/A	N/A	N/A
M15	Unknown mutation	8	6 (+1)	N/A	N/A	N/A
M16	G2447T	64	6 (+1)	N/A	N/A	N/A
M17	G2576T	32	7 (+2)	58.6	41.4	4:3
M38	G2576T	32	5	58.6	41.4	3:2
M18	N/A	4	6 (+1)	N/A	N/A	N/A
M19	Unknown mutation	8	6 (+1)	NA	NA	NA
M20	N/A	4	6 (+1)	N/A	N/A	N/A
M21	N/A	4	9 (+3)	N/A	N/A	N/A
M39	Unknown mutation	8	5	N/A	N/A	N/A

Cont.

Table 26. Pyrosequencing and hybridization results of all mutants selected during the course of five replicated experiments (cont.).

Strain	Mutation	Lin MIC (mg/L)	23S rRNA gene copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WTcopies
7717	**WT**	**4**	**5**	**WT**	**WT**	**WT**
M22	G2576T	8	6 (+1)	14.8	85.2	1:5
M23	G2576T	32	9 (+3)	45.4	54.0	4:5
M24	N/A	4	6 (+1)	N/A	N/A	N/A
M25	N/A	4	6 (+1)	N/A	N/A	N/A
M40	G2576T	32	6 (+1)	45.4	54.6	3:3
7501	**WT**	**4**	**5**	**WT**	**WT**	**WT**
M26	N/A	4	6 (+1)	N/A	N/A	N/A
M27	N/A	4	5	N/A	N/A	N/A
M28	N/A	4	5	N/A	N/A	N/A
M29	N/A	4	5	N/A	N/A	N/A
M41	N/A	4	5	N/A	N/A	N/A
M30	A2503G	8	6 (+1)	14.4	85.6	1:5
M31	N/A	4	4 (-1)	N/A	N/A	N/A
M32	N/A	4	5	N/A	N/A	N/A
M33	N/A	4	6 (+1)	N/A	N/A	N/A
M42	N/A	4	5	N/A	N/A	N/A

Figure 38. Hybridization to detect 23S rRNA gene copy number in linezolid-resistant mutants. Lanes 1 and 10 DIG-labelled ladder, lanes 2-5 M16-M19, lane 6 7500, lane 7 7501, lane 8 7499, lane 9 7717. 23S rRNA gene copy numbers for M17 (lane 3) are shown in white. Weak bands, an example of which are boxed, were not counted.



3.3.2 Replica plating

Mutants M26, M28 and M29 (all derived from isolate 7501) seemed to be in a transition period between erythromycin resistance and susceptibility, with some colonies still resistant to erythromycin whilst others were susceptible, resulting in a mixed population. Replica plating on to erythromycin (100 mg/L) enabled determination of the proportion of colonies still resistant to erythromycin and the proportion that were susceptible (see section 2.4.2). Replica plating was repeated three times for each isolate. The numbers of colonies on BHI, with no antibiotic added, were counted and compared with the numbers of colonies on BHI agar with 100 mg/L of erythromycin added. The percentage of erythromycin-resistant colonies was calculated and the average taken from the three replicated experiments. The average percentage of erythromycin-resistant colonies was 48.3%, 8.5% and 46.6% for M26, M28 and M29, respectively.

3.3.3 Summary

Linezolid resistance emerged quickest in mutants of the hypermutable strain, RN4220Δ*mutS*. Linezolid resistance was unstable in the majority of mutants, with mutants initially able to grow in the presence of 10 mg/L linezolid reverting back to linezolid susceptibility after a single passage on antibiotic-free medium. Growing erythromycin-resistant isolates in the presence of erythromycin and increasing concentrations of linezolid delayed the emergence of linezolid resistance in two of three strains, the exception being mutants of the hypermutable strain where the presence of erythromycin had no effect on the emergence of linezolid resistance. Growing erythromycin-resistant strains in the presence of lower concentrations of erythromycin, i.e., 8 mg/L as opposed to 100 mg/L and increasing concentrations of linezolid caused both increases and decreases in the time taken for resistance to emerge with little or no overall trend. There were no differences in the growth curves of erythromycin-resistant strains grown in the presence or absence of 100 mg/L erythromycin. Nearly half of the erythromycin-resistant isolates, grown in the absence of erythromycin, lost an *erm* determinant during selection of linezolid resistance, the majority losing an *erm*(C) determinant. No loss of *erm* determinants was seen with erythromycin-resistant strains grown in the presence of erythromycin and linezolid. A range of mutations were found in mutants,

with G2576T being the predominant one. Hybridization revealed that copy number in mutants ranged from one fewer to four more copies than the parent strain. Pyrosequencing and hybridization revealed mutants had between one and four mutated copies.

3.4 Selection of chloramphenicol-resistant mutants with cross-resistance to linezolid

Whilst growing linezolid-resistant mutants *in vitro*, some mutants were noticed with an elevated chloramphenicol MIC. The next section discusses these findings in greater detail and explores the possibility of cross-resistance between linezolid and chloramphenicol.

3.4.1 Characterization of chloramphenicol-resistant mutants

Whilst raising linezolid-resistant mutants *in vitro*, mutant ST/03/2121-M11 developed a T to C change at position 2504 in the genes encoding 23S rRNA, detected by sequencing and confirmed by a *Hin*1I PCR-RFLP. MIC determinations for this mutant revealed a four-fold increase in the chloramphenicol MIC, thought to be conferred by the T2504C mutation (see section 3.2, Table 16). Additionally, three of four mutants with G2576T mutations had 8-fold increases in chloramphenicol MICs. It was hypothesised that generating mutational resistance to chloramphenicol might select for cross-resistance to linezolid, and specifically for T2504C and G2576T mutations.

Mutants of RN4220, RN4220Δ*mutS*, ST/03/2121 and ST/03/2122 were selected in the presence of chloramphenicol, by passaging on agar containing increasing concentrations (in 2 mg/L intervals up to 16 mg/L) (see section 2.5.3). Mutants were passaged five times at 16 mg/L chloramphenicol to try to stabilise resistance. Mutants were designated by lettering A-E, prefixed by the number of the parent strain. Thirteen chloramphenicol-resistant mutants were selected, eight of which were cross-resistant to linezolid (Table 27). MICs of linezolid, chloramphenicol and erythromycin were determined for mutants (see section 2.3). No loss of erythromycin resistance was seen in any of the

Table 27. Chloramphenicol and linezolid MICs (mg/L) for parents and mutants of RN4220 (RN), RN4220Δ*mutS* (MS), ST/03/2121 (2121) and ST/03/2122 (2122). Mutants are designated by lettering A-E in the same colour as the parent strain.

Chloramphenicol MIC (mg/L)

Linezolid MIC (mg/L)	4	8	16	32	64	128	256
Lin^S 1	2122 A C E						
Lin^S 2		A D B C					
Lin^S 4		RN, MS	2121 B E B E	D			
Lin^R 8			A	B C C D			
Lin^R 16				D			
Lin^R 32				E			
Lin^R 64							A

mutants. In cross-resistant mutants chloramphenicol MICs ranged from 16 to >256 mg/L (parent strains 4 to 16 mg/L) and linezolid MICs from 8 to 64 mg/L (parent strains 1 to 4 mg/L). Resistance was unstable, especially in mutants of ST/03/2122, with three of five with chloramphenicol and linezolid MICs the same as their parent strain after a single passage on antibiotic-free agar. A positive correlation co-existed between chloramphenicol and linezolid MICs (Table 27).

Two mutants of RN4220, RN4220-A and RN4220-E, showed the highest chloramphenicol MICs. A 694 bp region of the 23S rRNA genes of these two mutants was DNA sequenced (see section 2.7.6), revealing T2500A and G2505A changes in RN4220-A and RN4220-E, respectively (Figure 39 [A] and [B]). The G2505A mutation was confirmed by PCR-RFLP with *Eco*RI-digested DNA (Figure 40) (see section 2.7.4) (Lobritz *et al.,* 2003). PCR-RFLP with *Nhe*I and *Hin*1I did not detect the G2576T and T2504C mutations in any of these mutants (see sections 2.7.4 and 2.7.5). All mutants underwent pyrosequencing to screen for seven mutations in the 23S rRNA genes: T2500A, A2503G, T2504C, G2505A, G2445T, G2447T, G2576T (see section 2.7.7). Pyrosequencing detected and quantified T2500A mutations in RN4220-A and RN4220Δ*mutS*-C and a G2505A mutation in RN4220-E (Table 28). No mutations were detected in any of the other mutants by PCR-RFLP, DNA sequencing or pyrosequencing.

Hybridization was used to determine the 23S rRNA gene copy number in parents and mutants (Figure 41[A] and [B]) (see section 2.8). 23S rRNA gene copy number varied between five and six copies. One mutant lost one gene copy, eight gained one gene copy and the remaining 11 had the same copy number as their parent strains. The pyrosequencing and hybridization data were combined to determine how many gene copies were mutated. RN4220-A, RN4220-E and RN4220Δ*mutS*-C all had one gene copy mutated. However, mutants RN4220-A and RN4220-E had chloramphenicol MICs of 256 mg/L and 32 mg/L, respectively. Resistance was highly unstable and some reversion to chloramphenicol and linezolid susceptibility occurred between MIC determinations and subsequent investigation of the number of mutated 23S rRNA gene copies by pyrosequencing and hybridization. Repeats of linezolid and chloramphenicol MICs confirmed

Figure 39. Chromatogram of the 23S rRNA genes of chloramphenicol-resistant mutants (A) Mutant RN4220-E with a G2505A mutation. **(B)** Mutant RN4220-A with a T2500A mutation.

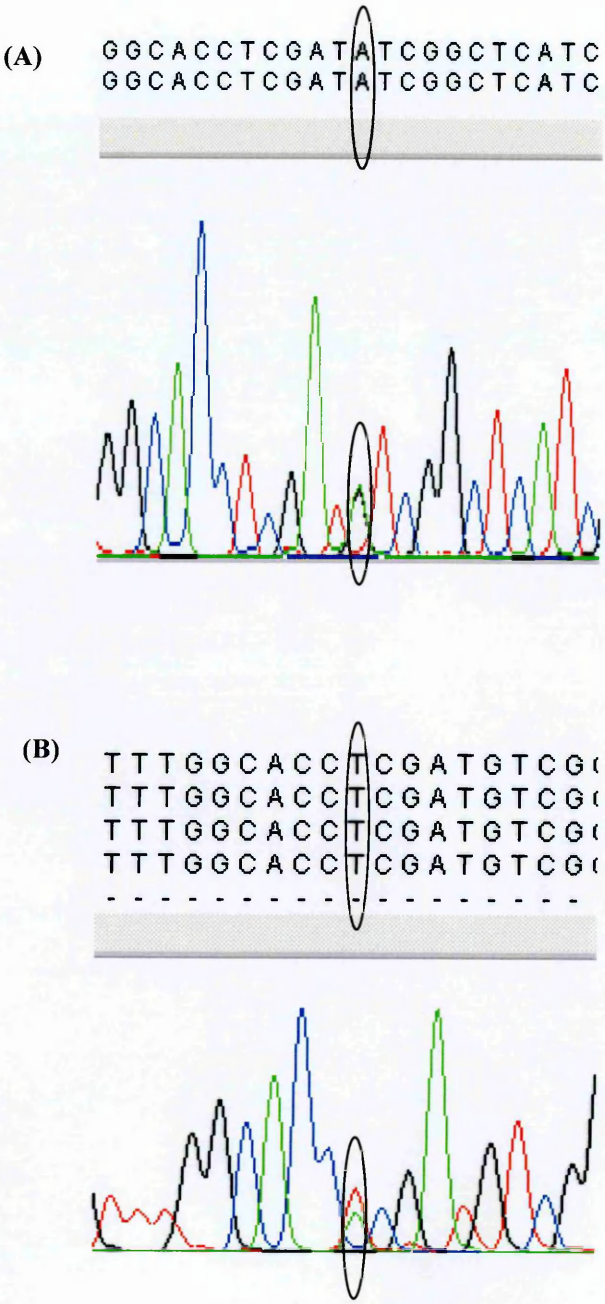


Figure 40. *Eco*RI digest to detect a G2505A mutation. Lane 1 homozygous G2505, lane 2 heterozygous G2505A, lane 3 water control, lane 4 123 bp ladder.

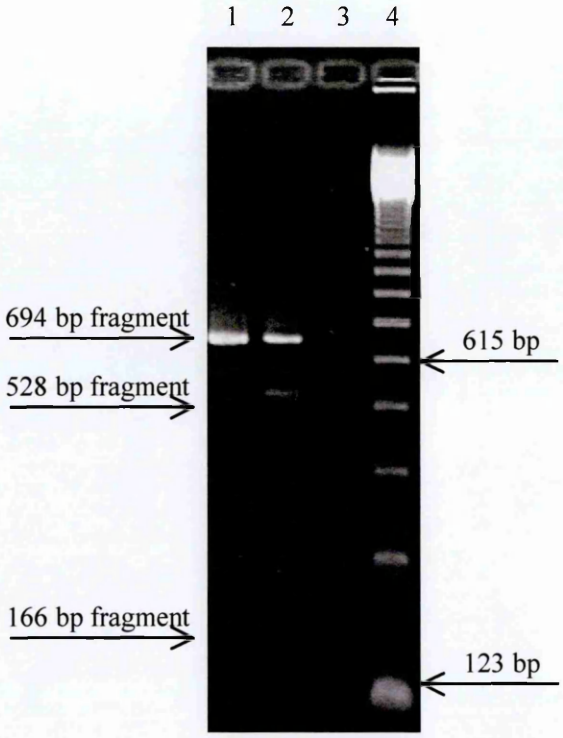


Table 28. Pyrosequencing and hybridization results for chloramphenicol-selected mutants. (See Appendix B Table 55 for a complete summary of pyrosequencing and hybridization results)

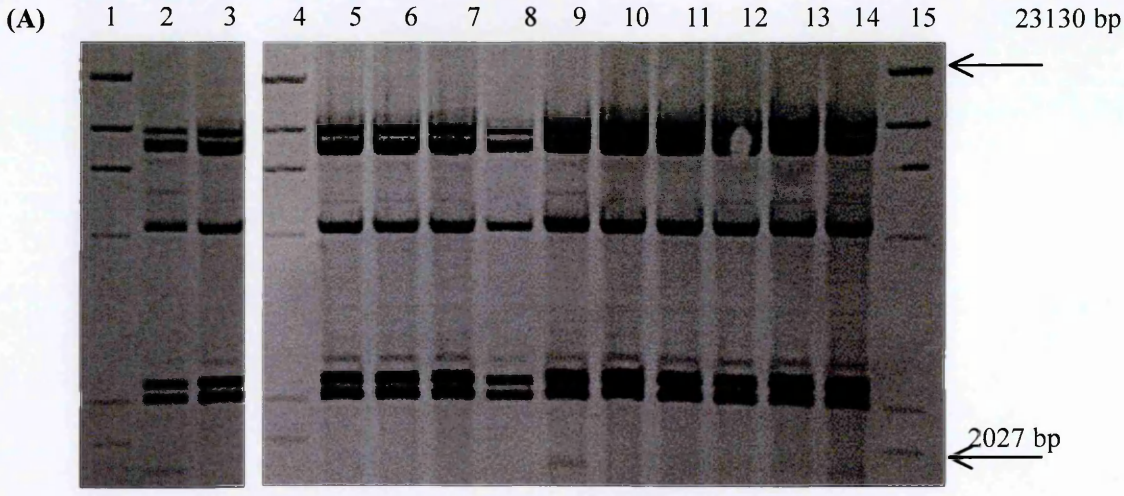
		Initial MICs (mg/L)		Repeated MICs (mg/L)				
Clone	**Mutation**	**Chlor**	**Lin**	**Chlor**	**Lin**	**23S rRNA gene copy no.**	**Av. % WT: mutated copies**	**No. WT: mutated copies**
RN4220	**N/A**	**8**	**4**	**8**	**4**	**6**	**N/A**	**N/A**
RN4220-A	T2500A	256	64	8	8	6	82.9 : 17.1	5 : 1
RN4220-B	N/A	32	8	2	4	6	N/A	N/A
RN4220-C	N/A	32	8	2	4	6	N/A	N/A
RN4220-D	N/A	32	16	2	4	5 (-1)	N/A	N/A
RN4220-E	G2505A	32	32	8	16	6	85.6 : 14.4	5 : 1
RN4220Δ*mutS*	**N/A**	**8**	**4**	**8**	**4**	**6**	**N/A**	**N/A**
RN4220Δ*mutS*-A	N/A	8	2	1	2	6	N/A	N/A
RN4220Δ*mutS*-B	N/A	16	4	2	4	6	N/A	N/A
RN4220Δ*mutS*-C	T2500A	32	8	8	8	6	83.3 : 16.7	5 : 1
RN4220Δ*mutS*-D	N/A	8	2	1	2	6	N/A	N/A
RN4220Δ*mutS*-E	N/A	16	4	1	4	6	N/A	N/A
ST/03/2121	**N/A**	**16**	**4**	**16**	**4**	**5**	**N/A**	**N/A**
ST/03/2121-A	N/A	16	8	2	4	6 (+1)	N/A	N/A

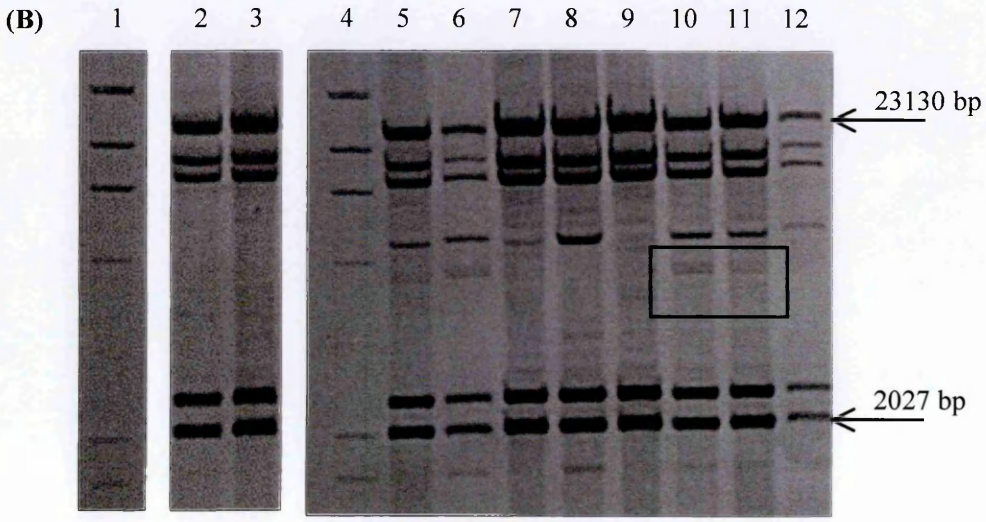
Cont.

Table 28. Pyrosequencing and hybridization results for chloramphenicol-selected mutants (cont.).

		Initial MICs (mg/L)		Repeated MICs (mg/L)				
Clone	**Mutation**	**Chlor**	**Lin**	**Chlor**	**Lin**	**23S rRNA gene copy no.**	**Av. % WT: mutated copies**	**No. WT: mutated copies**
ST/03/2121-B	N/A	16	4	1	4	6 (+1)	N/A	N/A
ST/03/2121-C	N/A	8	2	1	2	5	N/A	N/A
ST/03/2121-D	N/A	32	8	2	4	6 (+1)	N/A	N/A
ST/03/2121-E	N/A	16	4	2	4	5	N/A	N/A
ST/03/2122	**N/A**	**4**	**1**	**4**	**1**	**5**	**N/A**	**N/A**
ST/03/2122-A	N/A	4	1	1	1	6 (+1)	N/A	N/A
ST/03/2122-B	N/A	8	2	1	2	6 (+1)	N/A	N/A
ST/03/2122-C	N/A	4	1	1	1	6 (+1)	N/A	N/A
ST/03/2122-D	N/A	32	4	4	4	6 (+1)	N/A	N/A
ST/03/2122-E	N/A	4	1	1	1	6 (+1)	N/A	N/A

Figure 41. Hybridization of DNA from chloramphenicol-resistant mutants of RN4220, RN4220Δ*mutS*, ST/03/2121 and ST/03/2122 to detect 23S rRNA copy number. **(A)** Lanes 1, 4, 15 DIG-labelled ladder, lane 2 RN4220, lane 3 RN4220Δ*mutS*, lanes 5-9 RN4220 mutants A-E, lanes 10-14 RN4220Δ*mutS* mutants A-E. **(B)** Lanes 1, 4 and 12 DIG-labelled ladder, lane 2 ST/03/2121, lane 3 ST/03/2122, lanes 5-9 ST/03/2121 mutants A-E, lanes 10-11 RN4220Δ*mutS* mutants A-B. Weak bands, an example of which are boxed, were not counted.





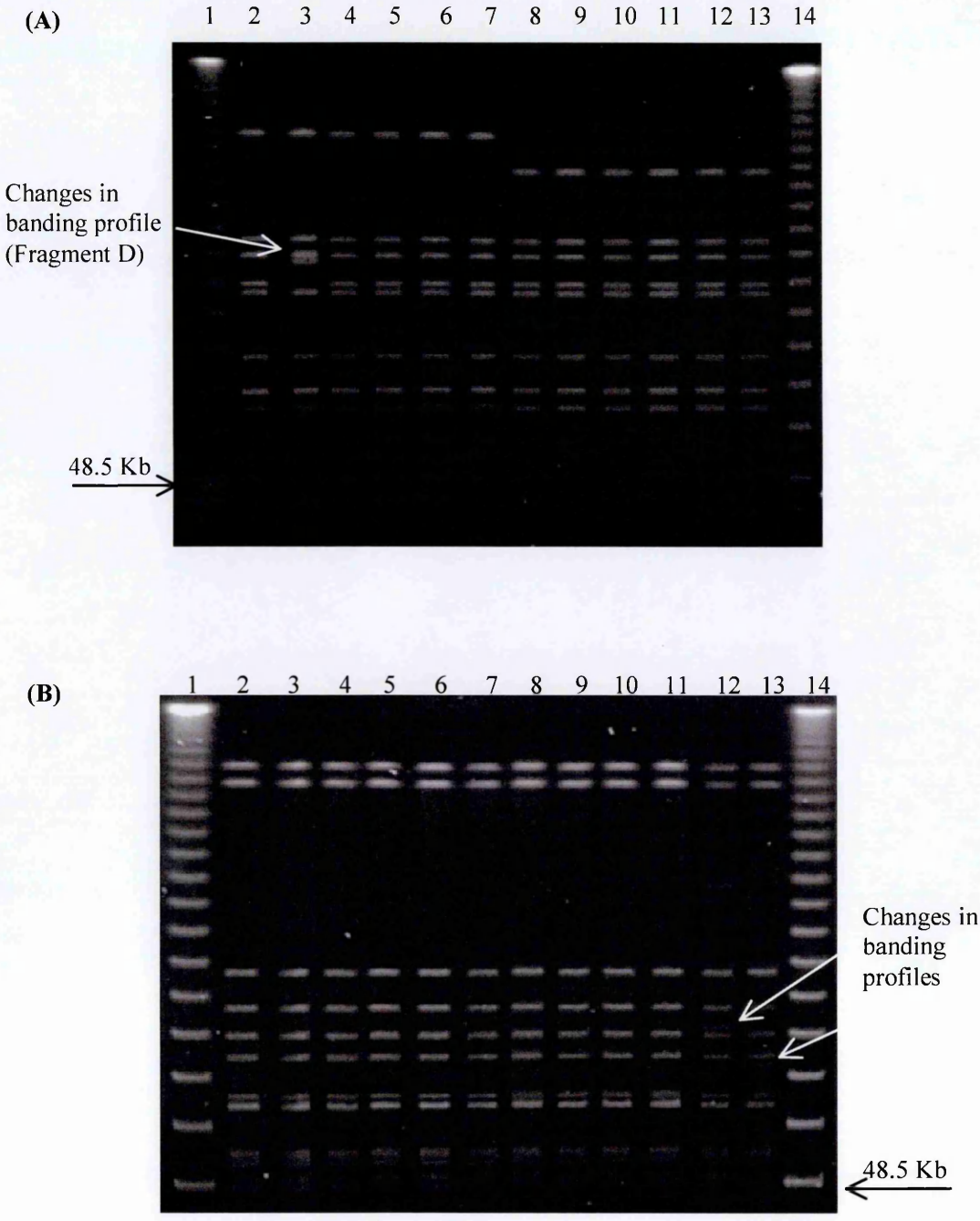
reversion towards susceptibility and were in agreement with pyrosequencing results (Table 28 and Appendix B Table 55). This reversion to susceptibility was further confirmed by mutants RN4220-A and RN4220-E having nearly equal numbers of mutated and wild-type copies present in their sequencing chromatograms (carried out before reversion towards susceptibility), however, only 17 and 14% of copies, respectively, are mutated according to the pyrosequencing result (carried out after reversion towards susceptibility had occurred).

Parentage of mutants was verified by PFGE (Figure 42 [A] and [B]). Mutants RN4220-A, ST/03/2122-D and ST/03/2122-E displayed a change in their PFGE banding profiles. On the PFGE profile of mutant RN4220-A there is an increase in the size of fragment D, possibly as a result of the loss of a *Sma*I site at one end of fragment D and its subsequent fusion with another fragment (Figure 42 [A] and see section 3.1.1 Figure 12). Fragment D was approximately 262 Kb in size and its adjacent fragment on the restriction digest map is fragment L, approximately 44 Kb in size (http://micro-gen.ouhsc.edu/s_aureus/s_aureus _home.htm). The two fragments have *rrn*A and *rrn*E operons at their junction. It is possible that changes in these regions due to exposure to chloramphenicol resulted in the loss of a *Sma*I site and fusion of the two fragments. However, this did not occur in all chloramphenicol-resistant mutants.

3.4.2 Summary

It was possible to raise chloramphenicol-resistant mutants that were cross-resistant to linezolid and there was a positive correlation between chloramphenicol and linezolid MICs for these mutants. Mutants of RN4220 produced high-level resistance to chloramphenicol. 23S rRNA mutations were only detectable in three of eight mutants cross-resistant to chloramphenicol and linezolid, the remainder had unstable resistance. These three mutants, with chloramphenicol MICs of ≥32 mg/L and linezolid MICs of ≥8 mg/L, harboured T2500A or G2505A mutations. No cross-resistant mutants were detected with T2504C and G2576T mutations. Changes in the number of genes encoding 23S rRNA were seen in nine of the 20 mutants, with loss or gain of one gene copy. Resistance was unstable with reversion to linezolid and chloramphenicol susceptibility in the

Figure 42. PFGE of *Sma*I-digested DNA from RN4220, RN4220Δ*mutS* and their chloramphenicol-resistant mutants. **(A)** Lanes 1 and 14, lambda ladder, lane 2 RN4220, lanes 3-7, RN4220 mutants A-E, lane 8 RN4220Δ*mutS* control, lanes 9-13, RN4220Δ*mutS* mutants A-E. Lane 3 shows changes in banding profile. **(B)** Lanes 1 and 14, lambda ladder, lane 2 ST/03/2121, lanes 3-7, ST/03/2121 mutants A-E, lane 8 ST/03/2122 control, lanes 9-13, ST/03/2122 mutants A-E. Lanes 12 and 13 show shifts in banding profiles.



absence of selection pressure, irrespective of mutation type. No loss of erythromycin resistance was associated with any of the mutants.

3.5 Analysis of linezolid-resistant clinical isolates

Clinical isolates suspected of linezolid resistance sent to ARMRL for susceptibility testing between 2003 and 2005 were characterized further.

3.5.1 Characterization of clinical linezolid-resistant isolates

Eleven suspected linezolid-resistant *S. aureus* isolates from five patients were submitted to ARMRL for susceptibility testing and confirmation of linezolid resistance (Table 29). Eight of the 11 isolates were from patients with cystic fibrosis. Most isolates were from the UK; exceptions were for 4a and 4b (Ireland) and 5a and 5b (Brazil) (Table 29).

An initial linezolid MIC, determined by agar dilution, established whether the MIC for each isolate was above the breakpoint (i.e., ≥4 mg/L). This was followed by an E Test to confirm the MIC (see section 2.3). The MICs for nine of 11 isolates were ≥16 mg/L. In the absence of treatment with linezolid the second isolate from patient 1 (1b) showed reversion to linezolid susceptibility, with an MIC of 0.5 mg/L. Its precursor isolate (1a) was linezolid-resistant, with an MIC of 16 mg/L. Isolate 5a was isolated at the beginning of therapy with linezolid (linezolid MIC 2 mg/L) and 5b was isolated later on during the course of therapy (linezolid MIC 32 mg/L).

Digestion of a 694 bp PCR amplicon of the 23S rRNA genes with the restriction endonuclease *Nhe*I revealed that all clinical linezolid-resistant isolates had a G to T mutation at position 2576 in the genes encoding 23S rRNA (see section 2.7.4). Seven of the nine were heterozygous for this mutation and two (4a and 4b) were homozygous. By *Hin*1I PCR-RFLP, none of the isolates had a T2504C mutation. Sequencing a 694 bp region of the 23S rRNA genes confirmed the presence of the G2576T mutation and the absence of other mutations. Pyrosequencing confirmed and quantified G2576T mutations in all linezolid-resistant isolates (Table 30) (see sections 2.7.6 and 2.7.7), revealing 35% to 100% of 23S rRNA gene copies were mutated. Isolates 4a and 4b had

Table 29. MICs (mg/L) for suspected linezolid-resistant clinical isolates.

Patient no.	Place of isolation	Site of isolation	Isolate	PFGE type	Mutation	Lin	Clin	Chlor	Ery	Fus	Oxa	Rif	Teic	Vanc
1a	Kent (UK)	Sputum (CF[A] patient)	H045360367	EMRSA-16	G2576T	16	>8	>8	>256	0.25	>16	>256	2	2
1b	Kent (UK)	Sputum (CF patient)	H053540293	EMRSA-16	WT	0.5	>8	>8	>256	0.25	>16	>256	2	1
2a	Kent (UK)	Sputum (CF patient)	H045360368	EMRSA-16	G2576T	32	>8	>8	>256	8	>16	>256	1	1
2b	Kent (UK)	Sputum (CF patient)	H053540294	EMRSA-16	G2576T	16	>8	>8	>256	8	>16	>256	1	1
3a	Nottingham (UK)	Swab from penis	H042800236	EMRSA-15	G2576T	16	16	>8	>256	0.125	>16	0.016	1	1
3b	Nottingham (UK)	Swab from drain site	H042800257	EMRSA-15	G2576T	32	16	>8	>256	0.125	>16	0.016	1	1
3c	Nottingham (UK)	Swab from perineum	H042800258	EMRSA-15	G2576T	32	16	>8	>256	0.125	>16	0.016	1	1
4a	Dublin (UK)	Sputum (CF patient)	H053760467	MSSA	G2576T	16	2	>8	>256	0.125	≤0.250	0.016	2	2
4b	Dublin (UK)	Sputum (CF patient)	H053760468	MSSA	G2576T	16	2	>8	>256	0.25	≤0.250	0.008	2	4
5a	Porto Alegre (Brazil)	Sputum (CF patient)	Brazilian 1	Brazilian MRSA	WT	2	>128	>8	>256	0.064	>16	64	2	2
5b	Porto Alegre (Brazil)	Sputum (CF patient)	Brazilian 2	Brazilian MRSA	G2576T	32	>128	>8	>256	2	>16	16	4	2

[A] Cystic fibrosis (CF)

Table 30. Percentage of mutated 23S rRNA genes, 23S rRNA copy number and number of mutated 23S rRNA gene copies in linezolid-resistant clinical isolates. (See Appendix B Table 55 for a complete summary of pyrosequencing and hybridization results)

Strain	Mutation	Lin MIC (mg/L)	23S rRNA gene copy no.	Av. % mutated copies	Av. % WT copies	No. mutated copies: No. WT copies
1a	G2576T	16	5	43.3	56.7	2:3
2a	G2576T	32	5	63.3	36.7	3:2
2b	G2576T	16	4	74.6	25.4	3:1
3a	G2576T	16	6	35.3	64.7	2:4
3b	G2576T	32	6	52.3	47.7	3:3
3c	G2576T	32	6	48.5	51.5	3:3
4a	G2576T	16	4	100.0	0.0	4:0
4b	G2576T	16	4	100.0	0.0	4:0
5b	G2576T	32	5	38.4	61.6	2:3

all copies mutated, however, their linezolid MICs were only 16 mg/L, which is low compared to several linezolid-resistant heterozygous isolates. Hybridization revealed 23S rRNA copy number to range from four to six among isolates. Isolates 4a and 4b produced an unusual fragment pattern with only four 23S rRNA gene copies (Figure 43). Combined, the pyrosequencing and hybridization data enabled the numbers of 23S rRNA copies mutated to be detected. Linezolid-resistant isolates had between two and four 23S rRNA copies mutated.

PFGE of the isolates, linezolid- susceptible and resistant, revealed three isolates to be EMRSA-15 (3a, 3b, 3c,), four to be EMRSA-16 (1a, 1b, 2a and 2b), two to be MSSA isolates (4a and 4b) and two to be the Brazilian endemic MRSA clone (5a, 5b) (Figure 44) (see section 2.6.3).

3.5.2 Mutation frequencies of linezolid-resistant clinical isolates

In order to see whether the linezolid-resistant clinical isolates were hypermutable, the frequency at which they generated mutants to rifampicin and fusidic acid at 4 x MIC was determined, unless resistance to these agents prevented this (Table 31) (see section 2.9.1). A mean mutation frequency was calculated from three replicated experiments.

There was an 86-fold difference in mutation frequencies to rifampicin between RN4220 and RN4220Δ*mutS* (Table 31, Figure 45 and Appendix B Table 53). RN4220, ST/03/2121, the MSSA control strain (H051000333) and the EMRSA-16 control strain (NCTC 13143) all had similar mutation frequencies to rifampicin. The EMRSA-15 control strain (NCTC-13142), had a mutation frequency five-fold greater than that of RN4220. A mutation frequency to rifampicin was only determinable for five of 11 isolates, as the remainder had high-level resistance to rifampicin. Clinical isolate ST/03/2122 also had high-level resistance to rifampicin. All the linezolid-resistant clinical isolates tested had mutation frequencies to rifampicin two- to seven-fold higher than that of RN4220, but 10 to 40-fold lower than RN4220Δ*mutS*.

RN4220Δ*mutS* had a mutation frequency to fusidic acid 18-fold higher than that of RN4220 (Table 31, Figure 46 and Appendix B Table 54). RN4220 and ST/03/2121 had similar mutation frequencies to each other. ST/03/2122 had a mutation frequency two-fold lower than RN4220.

Figure 43. Hybridization to determine 23S rRNA gene copy number in suspected linezolid-resistant clinical isolates. Lanes 1 and 15 DIG-labelled ladder, lane 2 RN4220, lane 3 RN4220Δ*mutS*, lane 4 ST/03/2121, lane 5 ST/03/2122, lane 6 5a, lane 7 5b, lane 8 1a, lane 9 2a, lane 10 3a, lane 11 3b, lane 12 3c, lane 13 4a, lane 14 4b. The unusual fragment pattern produced by isolates 4a and 4b is circled. Weak bands, an example of which are boxed, were not counted.

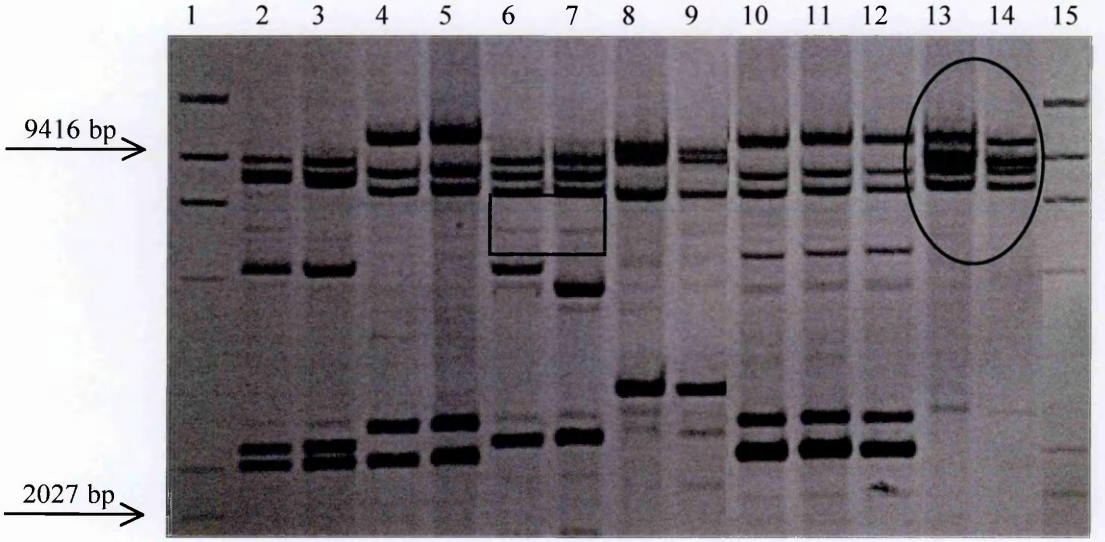
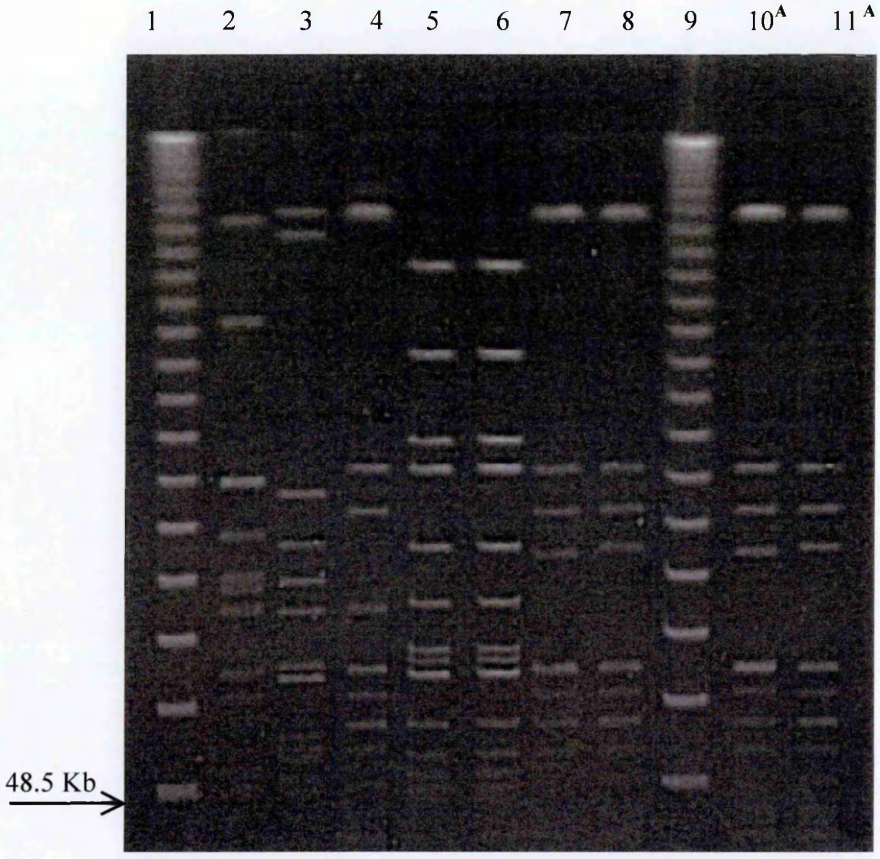


Figure 44. PFGE of representative linezolid-resistant clinical isolates. Lanes 1 and 9 48.5 Kb lambda ladder, lane 2 H051000333 (MSSA), lane 3 NCTC 13142 (EMRSA-15), lane 4 NCTC 13143 (EMRSA-16), lane 5 4a, lane 6 4b, lane 7 1b, lane 8 2b, lane 10 1a lane 11 2a.



[A] Variants of EMRSA-16

Table 31. Rifampicin and fusidic acid MICs (mg/L) and the concentrations of rifampicin and fusidic acid used in mutation frequency studies on linezolid-resistant clinical isolates (see Appendix B Table 53 and Table 54 for more detailed results).

Patient no.	Isolate	rrn mutation	Rif (MIC) (mg/L)	Rif 4 x MIC (mg/L)	Av. mutation frequency to rif and SD	Fus (MIC) (mg/L)	Fus 4 x MIC (mg/L)	Av. mutation frequency to fus and SD
N/A	**RN4220**	WT	0.016	0.064	$1.37 \pm 0.33 \times 10^{-8}$	0.125	0.5	$2.50 \pm 0.71 \times 10^{-7}$
N/A	**RN4220Δ*mutS***	WT	0.016	0.064	$1.18 \pm 0.41 \times 10^{-6}$	0.064	0.5	$6.33 \pm 3.84 \times 10^{-6}$
N/A	**ST/03/2121**	WT	0.016	0.064	$1.99 \pm 0.34 \times 10^{-8}$	0.125	0.5	$3.17 \pm 1.85 \times 10^{-7}$
N/A	**ST/03/2122**	WT	>256	N/A	N/A[A]	0.064	0.5	$1.59 \pm 0.49 \times 10^{-7}$
N/A	**H051000333 (MSSA)**	WT	0.004	0.016	$1.34 \pm 0.21 \times 10^{-8}$	16	64	$1.01 \pm 0.33 \times 10^{-8}$
N/A	**NCTC 13142 (EMRSA-15)**	WT	0.004	0.016	$6.17 \pm 4.65 \times 10^{-8}$	1	4	N/A[B]
N/A	**NCTC 13143 (EMRSA-16)**	WT	0.004	0.016	$1.32 \pm 0.75 \times 10^{-8}$	2	8	$1.18 \pm 0.84 \times 10^{-8}$
1a	H045360367	G2576T	>256	N/A	N/A[A]	0.25	1	$1.42 \pm 0.22 \times 10^{-7}$
1b	H053540293	WT	>256	N/A	N/A[A]	0.25	1	$2.66 \pm 2.05 \times 10^{-8}$
2a	H045360368	G2576T	>256	N/A	N/A[A]	8	32	$3.04 \pm 1.57 \times 10^{-8}$
2b	H053540294	G2576T	>256	N/A	N/A[A]	8	32	$5.98 \pm 1.38 \times 10^{-9}$
3a	H042800236	G2576T	0.016	0.064	$5.21 \pm 2.83 \times 10^{-8}$	0.125	0.5	$9.24 \pm 3.31 \times 10^{-7}$
3b	H042800257	G2576T	0.016	0.064	$9.70 \pm 5.40 \times 10^{-8}$	0.125	0.5	$9.99 \pm 6.02 \times 10^{-7}$
3c	H042800258	G2576T	0.016	0.064	$3.87 \pm 0.73 \times 10^{-8}$	0.125	0.5	$1.50 \pm 0.98 \times 10^{-6}$

Cont.

Table 31. Rifampicin and fusidic acid MICs (mg/L) and the concentrations of rifampicin and fusidic acid used in mutation frequency studies on linezolid-resistant clinical isolates (cont.).

Patient no.	Isolate	rrn mutation	Rif (MIC)	Rif 4 x MIC	Av. mutation frequency to rif and SD	Fus (MIC)	Fus 4 x MIC	Av. mutation frequency to fus and SD
4a	H053760467	G2576T	0.016	0.064	$3.15 \pm 1.73 \times 10^{-8}$	0.25	1	$8.17 \pm 3.66 \times 10^{-9}$
4b	H053760478	G2576T	0.016	0.064	$4.01 \pm 1.36 \times 10^{-8}$	8	32	$7.10 \pm 1.40 \times 10^{-9}$
5a	Brazilian 1	WT	64	256	N/A[A]	0.064	0.25	$5.87 \pm 2.40 \times 10^{-8}$
5b	Brazilian 2	G2576T	16	64	N/A[A]	2	8	$3.38 \pm 3.28 \times 10^{-7}$

[A] Isolate resistant to rifampicin.

[B] Mutation frequency below the detectable limit.

Figure 45. Mutation frequencies of linezolid-resistant clinical isolates to rifampicin. Each dot represents the mean mutation frequency for each strain, along with the range. The upper range of RN4220 is signified with a dotted line (above this line is designated 'Elevated'). The lower range of RN220Δ*mutS* is signified with a dotted line (above this line is designated 'Hypermutable').

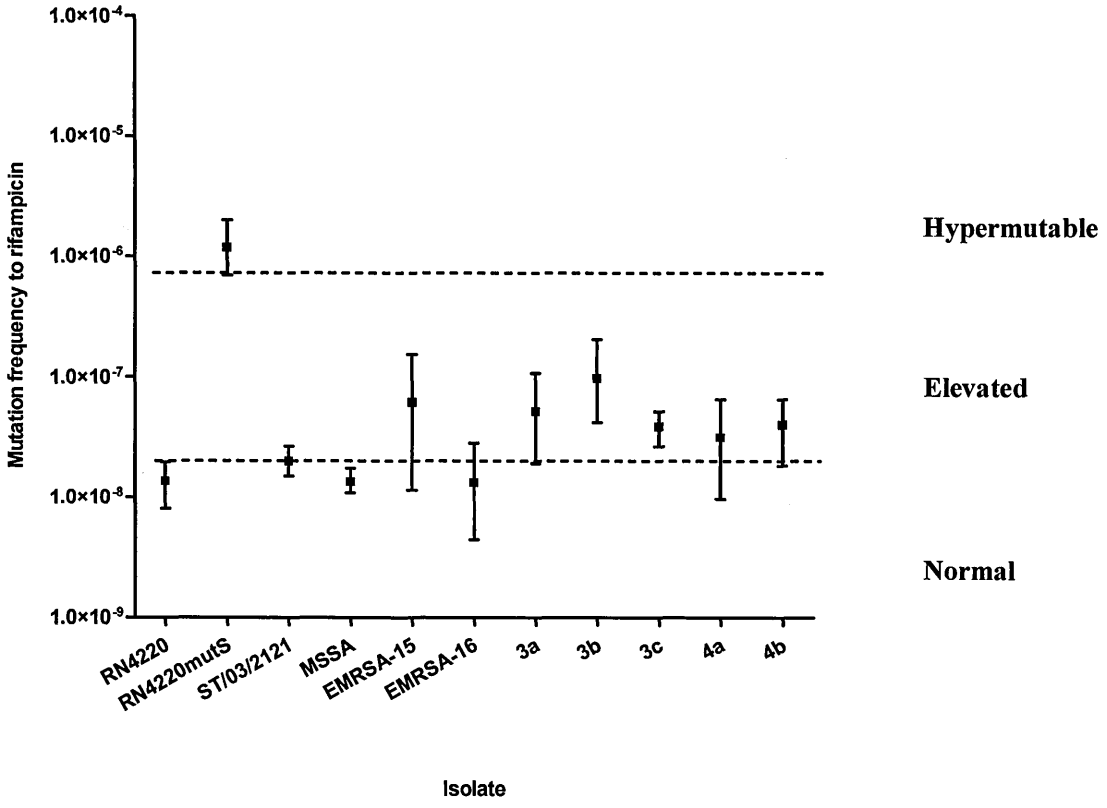
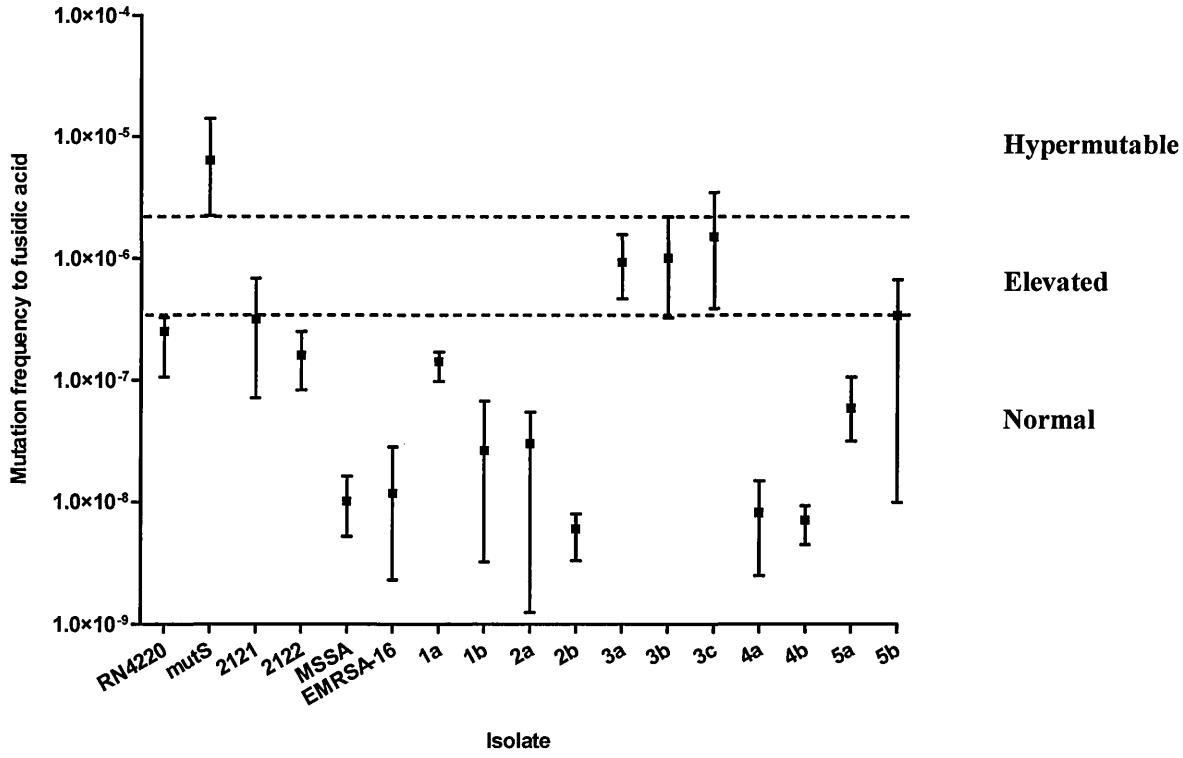


Figure 46. Mutation frequencies of linezolid-resistant clinical isolates to fusidic acid. Each dot represents the mean mutation frequency for each strain, along with the range. The upper range of RN4220 is signified with a dotted line (above this line is designated 'Elevated'). The lower range of RN220Δ*mutS* is signified with a dotted line (above this line is designated 'Hypermutable').



The MSSA (H051000333) and EMRSA-16 (NCTC 13143) control strains had mutation frequencies 25-fold and 21-fold lower than RN4220, respectively. The control strain, EMRSA-15 (NCTC 13412), mutated at a level below the detectable limit. A mutation frequency was detectable for all clinical isolates. Four of the clinical isolates had mutation frequencies higher than RN4220, the remaining six had mutation frequencies lower than RN4220. The isolate 1b, isolated from the same patient approximately six months after 1a, had a mutation frequency to fusidic acid 12-fold higher than its precursor. However, both had mutation frequencies lower than RN4220. Isolates 2a and 2b, isolated sequentially from the same patient, had similar mutation frequencies, both of which were lower than that of RN4220. Isolates 4a and 4b, isolated sequentially from the same patient, had mutation frequencies 31- and 35-fold lower than RN4220. The linezolid-resistant isolate 5b had a mutation frequency six-fold greater than its linezolid-susceptible precursor, 5a. Three sequential, clinical isolates from the same patient (3a, 3b, 3c) had mutation frequencies four- to six-fold higher than RN4220. These three isolates were the only ones that displayed increased mutation frequencies to rifampicin and fusidic acid.

3.5.3 Fosfomycin disc tests

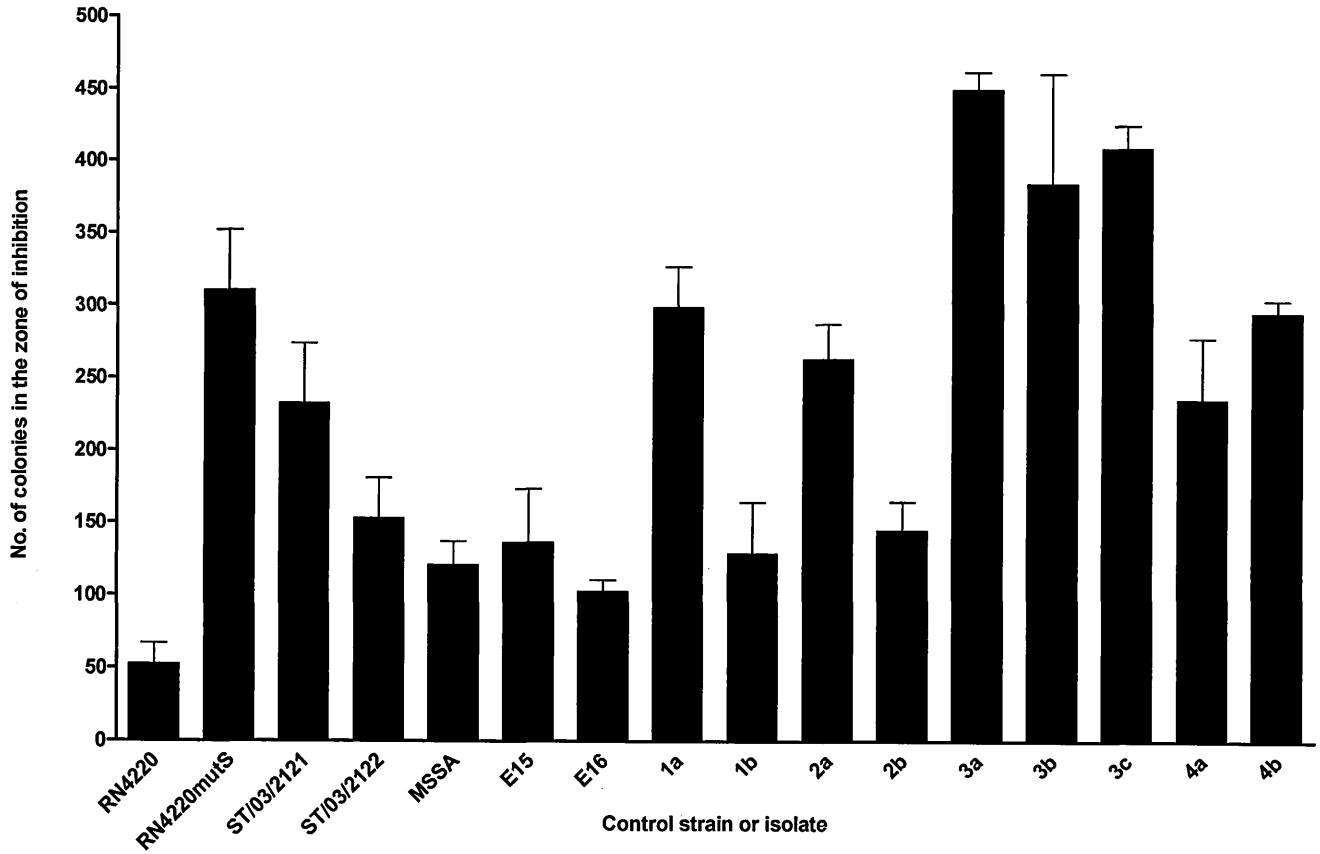
This method was used as a screen for hypermutators (see section 2.9.2). The number of colonies in the zone of clearing were counted and the zone diameter measured. The average of three repeated experiments was calculated.

The number of colonies in the zone of clearing for control strains was as for section 3.2.6. Briefly, RN4220Δ*mutS* produced an average five-times that of RN4220 (Table 32 and Figure 47). ST/03/2121 and ST/03/2122 produced averages four- and three-times that of RN4220, respectively. Isolates 5a and 5b were resistant to fosfomycin, all other clinical isolates were tested. Five isolates had between three and 158 more colonies in the zone of clearing than RN4220Δ*mutS*. Of these five, three were from the same patient (3a, 3b, 3c) and had previously displayed mutation frequencies to rifampicin and fusidic acid higher than those for RN4220. Four isolates had between 57 and 163 fewer colonies in the zone of clearing than RN4220Δ*mutS*, but still more than

Table 32. Number of colonies in the zone of clearing, and zone diameter produced by linezolid-resistant clinical isolates around fosfomycin discs (50 µg).

Strain	Mutation	Linezolid MIC (mg/L)	No. of colonies in zone			Average ± SD	Zone diameter (mm)			Average ± SD
RN4220	N/A	4	66	53	59	**60 ± 9**	41.8	42.0	43.7	**42.5 ± 0**
RN4220Δ*mutS*	N/A	4	302	267	306	**292 ± 25**	43.8	43.8	44.3	**43.9 ± 0**
ST/03/2121	N/A	4	276	243	234	**251 ± 23**	42.4	41.3	42.4	**42.0 ± 1**
ST/03/2122	N/A	1	128	148	183	**153 ± 14**	39.3	40.2	39.4	**39.6 ± 1**
1a	G2576T	16	269	303	325	**299 ± 24**	39.8	42.1	39.4	**40.5 ± 2**
1b	G2576T	0.5	138	159	89	**129 ± 15**	37.3	35.9	32.5	**35.2 ± 1**
2a	G2576T	32	266	286	237	**263 ± 14**	49.5	47.4	46.6	**47.8 ± 1**
2b	G2576T	16	132	135	168	**145 ± 2**	48.2	50.3	51.0	**49.8 ± 1**
3a	G2576T	16	456	458	436	**450 ± 1**	44.2	43.5	47.0	**44.9 ± 0**
3b	G2576T	32	315	466	374	**385 ± 107**	44.6	44.4	44.5	**44.5 ± 0**
3c	G2576T	32	401	399	428	**409 ± 1**	48.6	46.1	46.0	**46.9 ± 2**
4a	G2576T	16	202	221	283	**235 ± 13**	42.6	43.0	42.2	**42.6 ± 0**
4b	G2576T	16	288	293	305	**295 ± 4**	44.9	44.2	45.6	**44.9 ± 0**

Figure 47. Number of colonies in the zone of inhibition of a fosfomycin disc (50 μg) produced by linezolid-resistant clinical isolates. Standard deviations are represented by error bars.



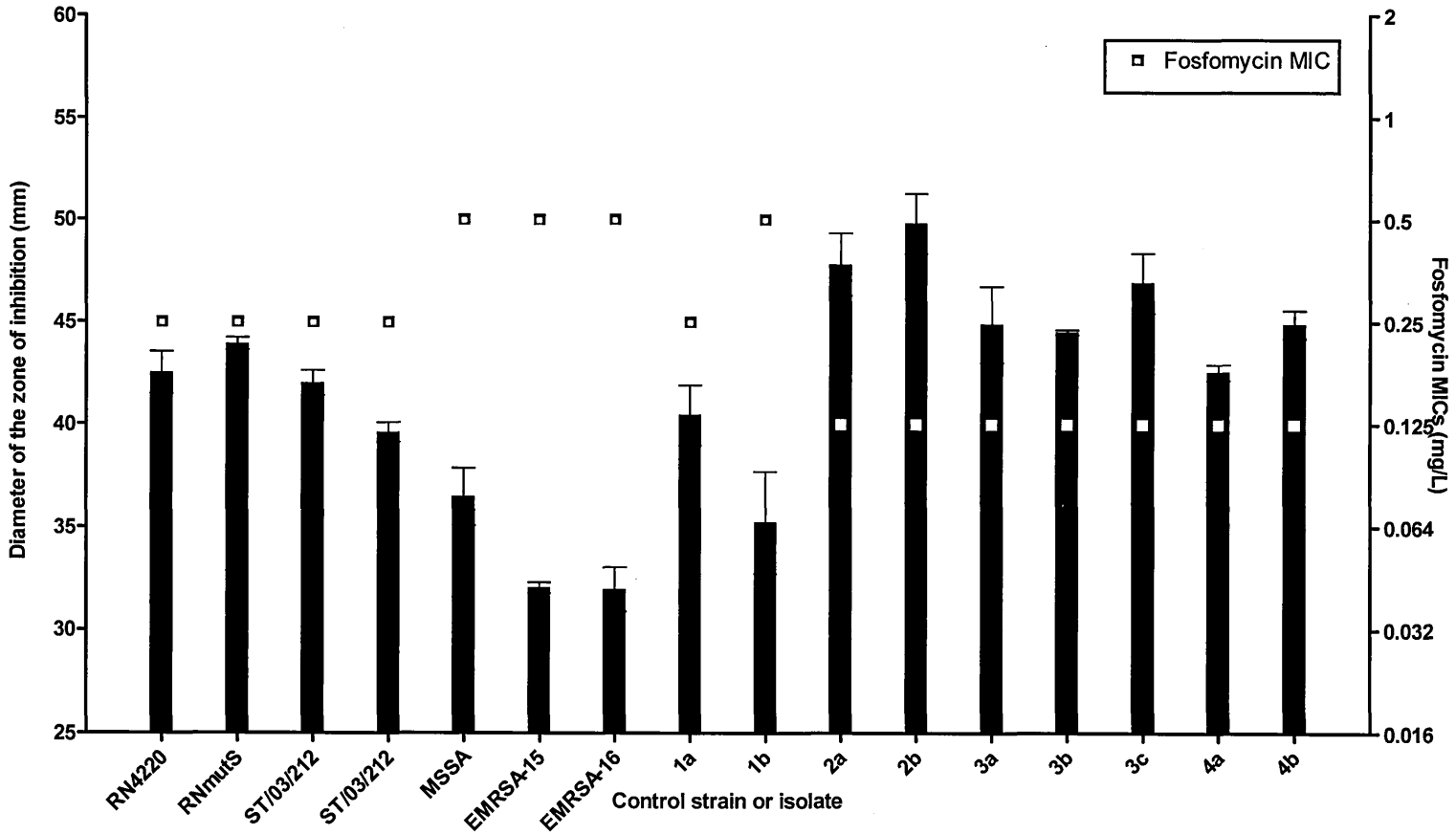
produced by RN4220. Isolate 1a had, on average, 171 more colonies in the zone of clearing than the subsequent isolate (1b). Isolate 2a had, on average, 118 more colonies less in the zone of clearing than the subsequent isolate (2b). Isolates 3a, 3b and 3c had, on average, 158, 93 and 117 more colonies in the zone of clearing than RN4220Δ*mutS*.

Three mutants had a zone diameter smaller than that produced by RN4220Δ*mutS*, one of which had more colonies in the zone of clearing (1a) (Table 32 and Figure 48). The remaining six all had larger zone diameters, with four of these producing more colonies in the zone, than RN4220Δ*mutS*. Fosfomycin MICs related to zone diameters, with seven out of eight strains tested with an MIC of 0.25 mg/L. Results showed a high fosfomycin MIC is not necessarily a prerequisite for a greater number of colonies appearing in the zone of clearing.

3.5.4 Summary

Nine out of 11 isolates were resistant to linezolid, with MICs ≥16 mg/L. All were positive for the G2576T mutation. Two were homozygous for the G2576T mutation, the remainder were heterozygous. No additional mutations were found in the 694 bp region of the 23S rRNA genes sequenced. The 23S rRNA copy number varied from four to six and the number of mutated copies ranged from two to four. Two isolates of the same strain had all four 23S rRNA gene copies mutated, yet only with a linezolid MIC of 16 mg/L. Mutation frequencies were difficult to ascertain with the majority of isolates being resistant to rifampicin. RN4220Δ*mutS* mutated at the greatest frequency to both rifampicin and fusidic acid. All the clinical isolates, that were able to be tested, had a mutation frequency to rifampicin greater than RN4220. However, only four isolates had mutation frequencies to fusidic acid greater than RN4220, and none so high as RN4220Δ*mutS*. Only three isolates had elevated mutation frequencies to both rifampicin and fusidic acid when compared with RN4220. The same three isolates had more colonies in the zone of inhibition surrounding a fosfomycin disc than the hypermutable strain RN4220Δ*mutS*.

Figure 48. Diameter of the zone of inhibition surrounding a fosfomycin disc (50 μg) produced by linezolid-resistant clinical isolates. Standard deviations are represented by error bars.



3.6 Determination of 23S rRNA copy number in linezolid-resistant isolates and mutants

The next section summarises the results from pyrosequencing and hybridization experiments to determine the number of mutated 23S rRNA gene copies in linezolid-resistant isolates from the clinic and laboratory-selected mutants and the relationship between this and linezolid MIC.

3.6.1 Pyrosequencing

Pyrosequencing was used to detect and quantify the percentage of mutated 23S rRNA gene copies in clinical linezolid-resistant isolates, laboratory-selected mutants, and their parent strains (see section 2.7.7).

A 364 bp region of the 23S rRNA genes was amplified (Figure 49). Three pyrosequencing primers were then used to detect the presence of seven known mutations; T2500A (Figure 50), A2503G (Figure 51), T2504C (Figure 52), G2505A (Figure 53), G2445T (not shown), G2447T (Figure 54) and G2576T (Figure 55). Quantification of the percentage of mutated copies was not possible for G2445T and G2447T mutations due to their presence within a homopolymer of four G nucleotides which the software found difficult to distinguish. A mutant with a G2445T mutation was not available to validate the assay. See relevant sections (3.2, 3.2.7, 3.4, 3.5) for detailed analysis of pyrosequencing results and Appendix B Table 55.

In total, 103 *S. aureus* isolates were pyrosequenced; 11 control strains, 46 linezolid-resistant mutants, 35 linezolid-susceptible isolates and 11 suspected linezolid-resistant clinical isolates. Mutations were successfully detected in 50 mutants. Mutations were detected in 41 of 46 *in vitro*-selected mutants; 16 had G2576T, 13 had G2447T, seven had T2504C, two had T2500A, one had a A2503G mutation and one had a G2505A mutation (Table 33 and Appendix B Table 55). One mutant had two mutations, G2576T and A2503G. All other mutations were present singularly. Mutations could not be detected in five laboratory-selected mutants (linezolid MICs 8-16 mg/L).

Figure 49. PCR to amplify 364 bp of the 23S rRNA genes. Lane 1 123 bp ladder, lanes 2-9 364 bp PCR product, lane 10 water control.

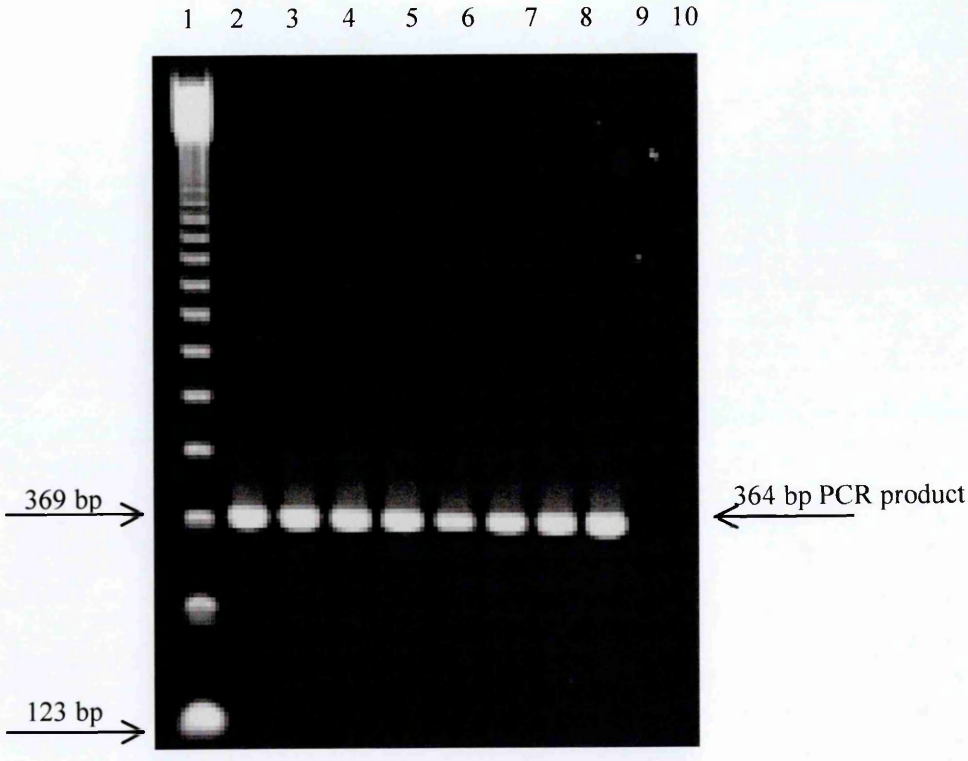
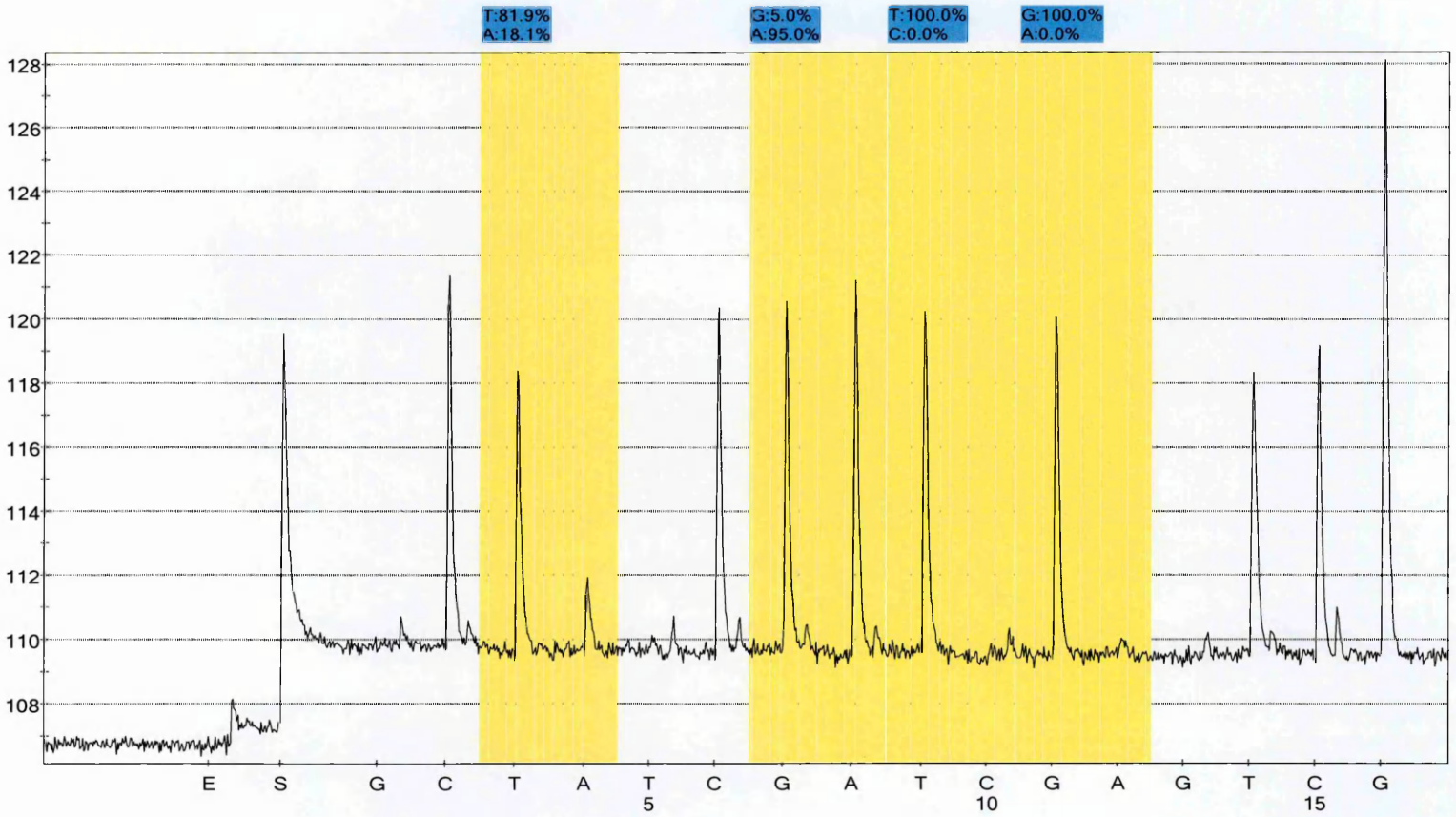
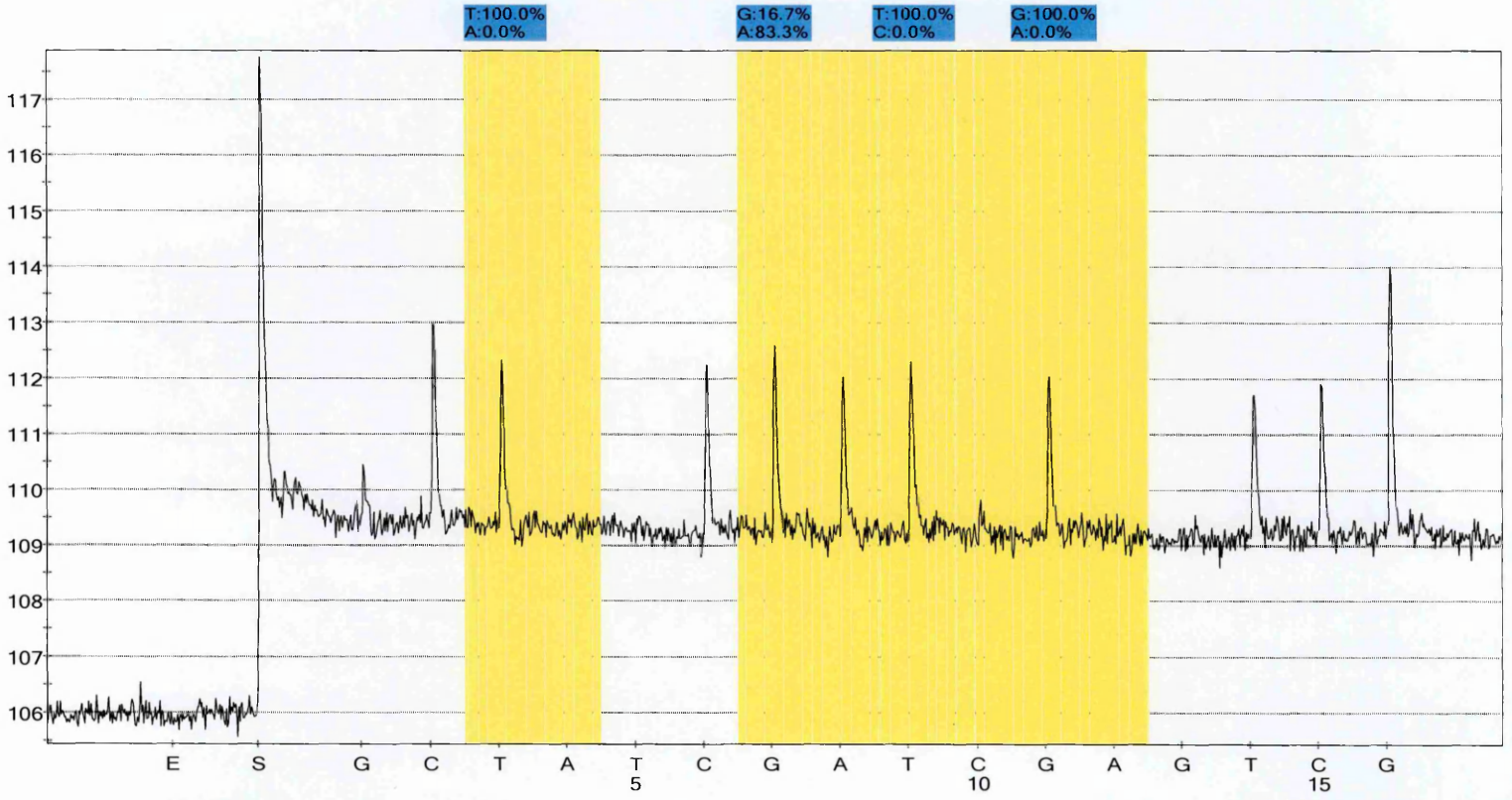


Figure 50. Pyrogram of a T2500A mutation in mutant RN4220-A. The presence of a wild-type T peak and a mutant A peak, at position 2500, indicates heterozygosity. Mutant negative for A2503G, T2504C and G2505A mutations.



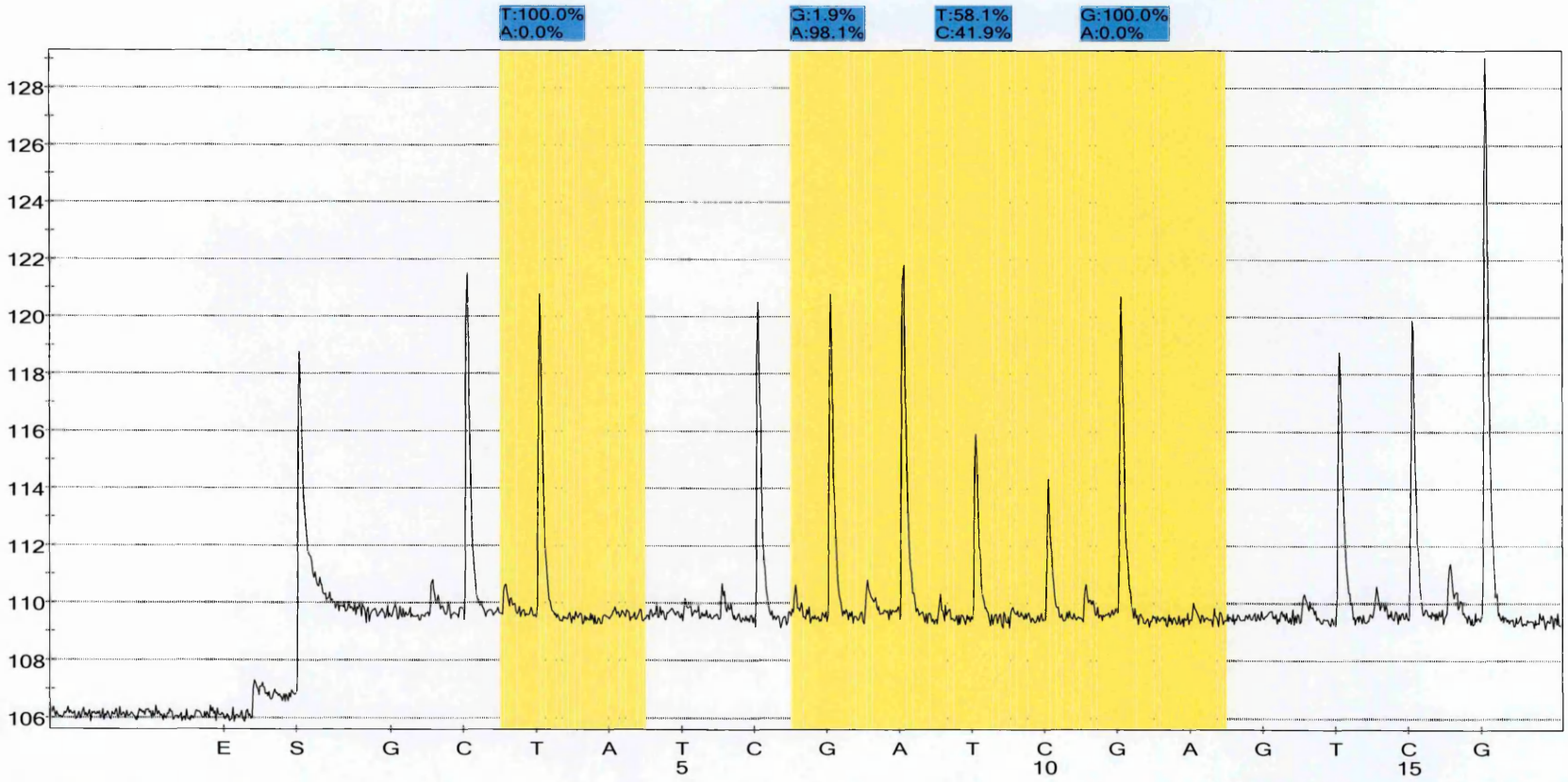
Sequence sought: C(T/A)CG(A/G)(T/C)(G/A)TCGG **Actual sequence:** C(T/A)CGATGTCGG

Figure 51. Pyrogram of a A2503G mutation in mutant M30. The presence of a wild-type A peak and a mutant G peak, at position 2503, indicates heterozygosity. Mutant negative for T2500A, T2504C and G2505A mutations.



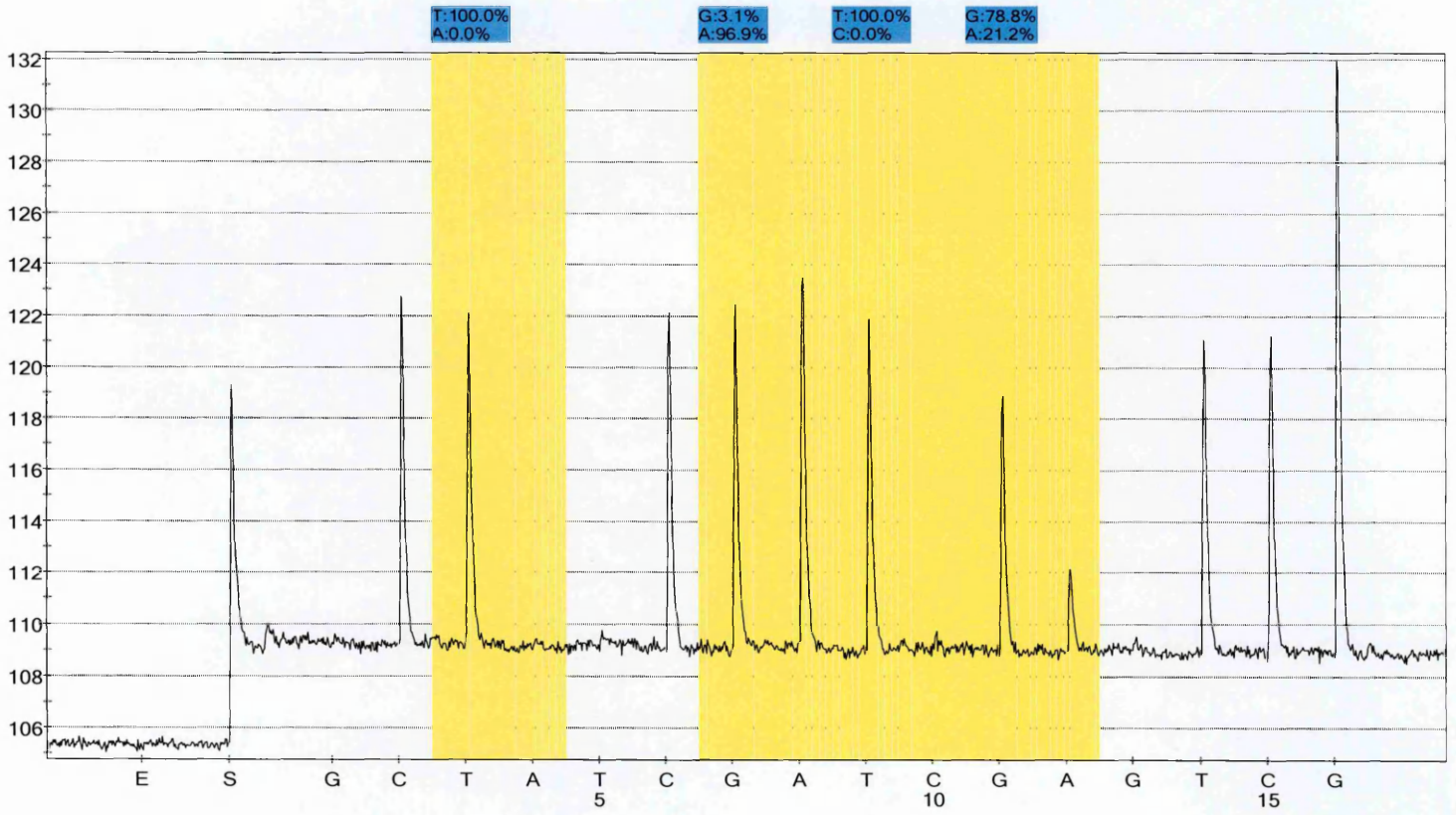
Sequence sought: C(T/A)CG(A/G)(T/C)(G/A)TCGG **Actual sequence:** CTCG(A/G)TGTCGG

Figure 52. Pyrogram of a T2504C mutation in mutant M12. The presence of a wild-type T peak and a mutant C peak, at position 2504, indicates heterozygosity. Mutant negative for T2500A, A2503G and G2505A mutations.



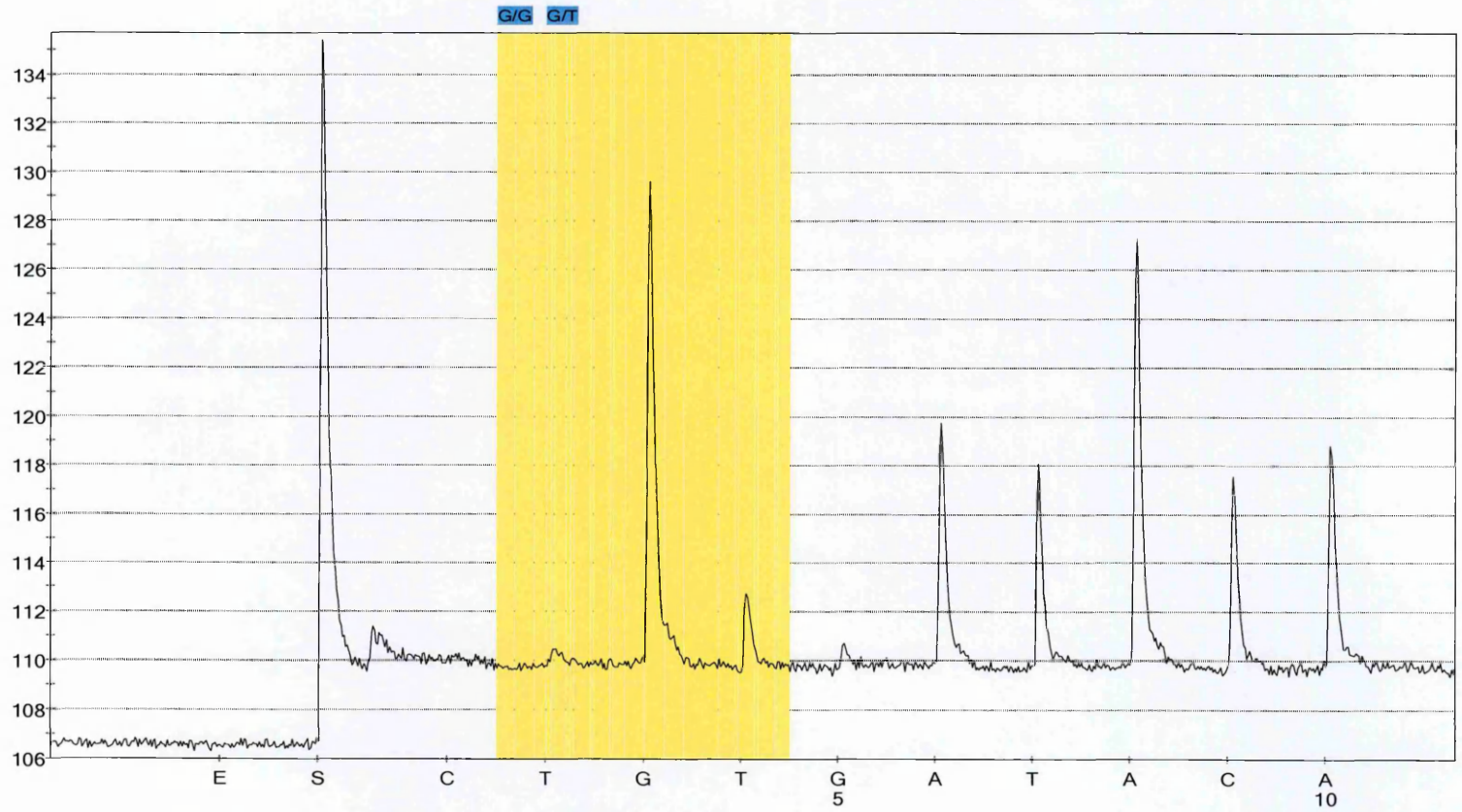
Sequence sought: C(T/A)CG(A/G)(T/C)(G/A)TCGG **Actual sequence:** CTCGAT/CGTCGG

Figure 53. Pyrogram of a G2505A mutation in mutant RN4220-E. The presence of a wild-type T peak and a mutant C peak, at position 2504, indicates heterozygosity. Mutant negative for T2500A, A2503G and G2505A mutations.



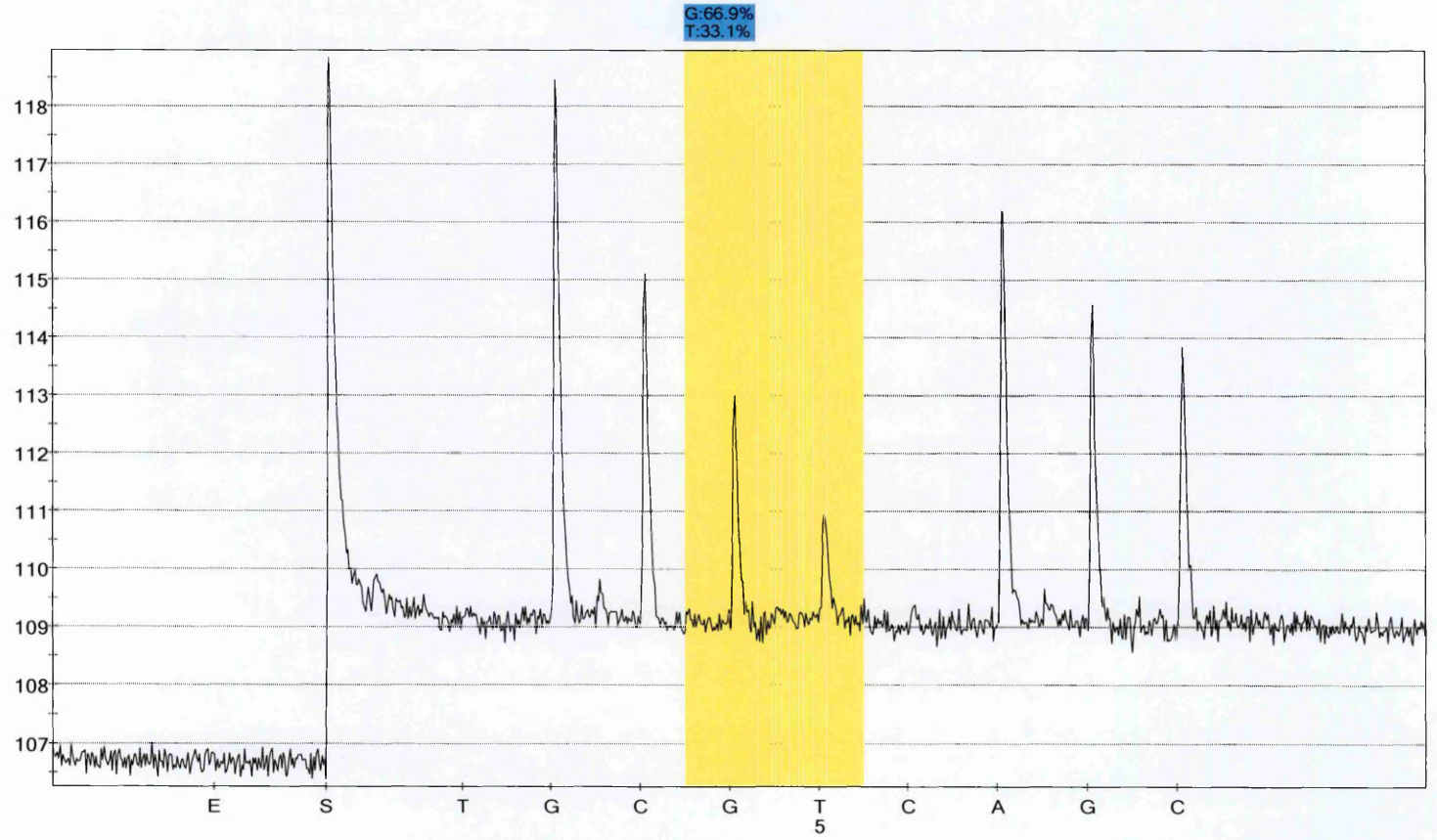
Sequence sought: C(T/A)CG(A/G)(T/C)(G/A)TCGG **Actual sequence:** CTCGAT(G/A)TCGG

Figure 54. Pyrogram of a G2447T mutation in mutant RN4220-M3. The presence of a wild-type G peak and a mutant T peak, at position 2447, indicates heterozygosity. Mutant negative for G2445T mutation.



Sequence sought: (G/T)G(G/T)ATAACA **Actual sequence:** GG(G/T)ATAACA

Figure 55. Pyrogram of a G2576T mutation in RN4220Δ*mutS*-M7. The presence of a wild-type G peak and a mutant T peak, at position 2576, indicates heterozygosity.



Sequence sought: GC(G/T)AGC **Actual sequence:** GC(G/T)AGC

G2576T mutations were detected in nine of 11 suspected linezolid-resistant clinical isolates. Two isolates were linezolid susceptible.

Linezolid-resistant clinical isolates had 35-100% of 23S rRNA gene copies mutated, the majority had 40-50% of copies mutated. Laboratory-selected mutants with a G2576T mutation had between 15% and 65% of gene copies mutated, the majority of mutants had 40-50% of copies mutated. Laboratory-selected mutants with a T2504C mutation had between 13 and 48% of gene copies mutated, the majority had 30-40% of copies mutated and mutants with T2500A, A2503G and G2505A mutations all had 10-20% of copies mutated. Regardless of mutation, only laboratory-selected mutants had between 10 and 20% of 23S rRNA copies mutated, all linezolid-resistant clinical isolates had over 35% of copies mutated (Table 33) (see sections 3.2, 3.2.7, 3.4, 3.5 for a more detailed analysis of pyrosequencing results).

Figure 56 illustrates the positive correlation between linezolid MIC and the percentage of 23S rRNA copies mutated; an increased percentage of mutated 23S rRNA copies leads to an increased linezolid MIC. There were exceptions to this trend. Two clinical isolates, 4a and 4b, had four 23S rRNA gene copies, all mutated, yet only a linezolid MIC of 16 mg/L. Another exception was clinical isolate, 2b, which had 75% of copies mutated but only a linezolid MIC of 16 mg/L.

3.6.2 Hybridization

Hybridization was used to determine the number of genes encoding 23S rRNA in linezolid-resistant clinical isolates, laboratory-selected linezolid-resistant and -susceptible isolates and parent strains (see individual results chapters for more details) (see section 2.8). The same 103 isolates and mutants that were pyrosequenced underwent hybridization to detect 23S rRNA gene copy number. The 23S rRNA gene copy number in control isolates was five or six, with the majority having five copies (Table 34 and Appendix B Table 55). The 23S rRNA gene copy number in laboratory-selected mutants, both linezolid-resistant and -susceptible (81), ranged from four to nine with 52 (64%) having six copies. In linezolid-resistant clinical isolates copy number ranged from four to six, with equal numbers of isolates having four, five and six copies. Of 46 laboratory-selected linezolid-resistant mutants, 25 mutants had no changes and 21 mutants had

Table 33. Percentage of mutated 23S rRNA copies in all linezolid-resistant isolates and mutants. (See Table 17, Table 19, Table 26, Table 28, Table 30 and Table 55 for individual results)

% of 23S rRNA copies mutated	G2447T	G2576T	T2500A	A2503G	T2504C	G2505A
0	13[A]	–	–	–	–	–
10-20	–	1	2	1	1	1
20-30	–	–	–	–	–	–
30-40	–	5	–	1[B]	4	–
40-50	–	9	–	–	2	–
50-60	–	5	–	–	–	–
60-70	–	3[B]	–	–	–	–
70-80	–	1	–	–	–	–
80-90	–	–	–	–	–	–
90-100	–	2	–	–	–	–
Total	**13**	**26**	**2**	**2**	**7**	**1**

[A] The percentage of mutated 23S rRNA copies could not be determined for G2445T and G2447T mutations due to their presence within a homopolymer of four G nucleotides. [B] Mutant RN4220Δ*mutS*-M9a has two mutations, G2576T and A2503G.

Figure 56. Percentage of mutated 23S rRNA gene copies versus linezolid MIC (mg/L). The different 23S rRNA mutations found are indicated.

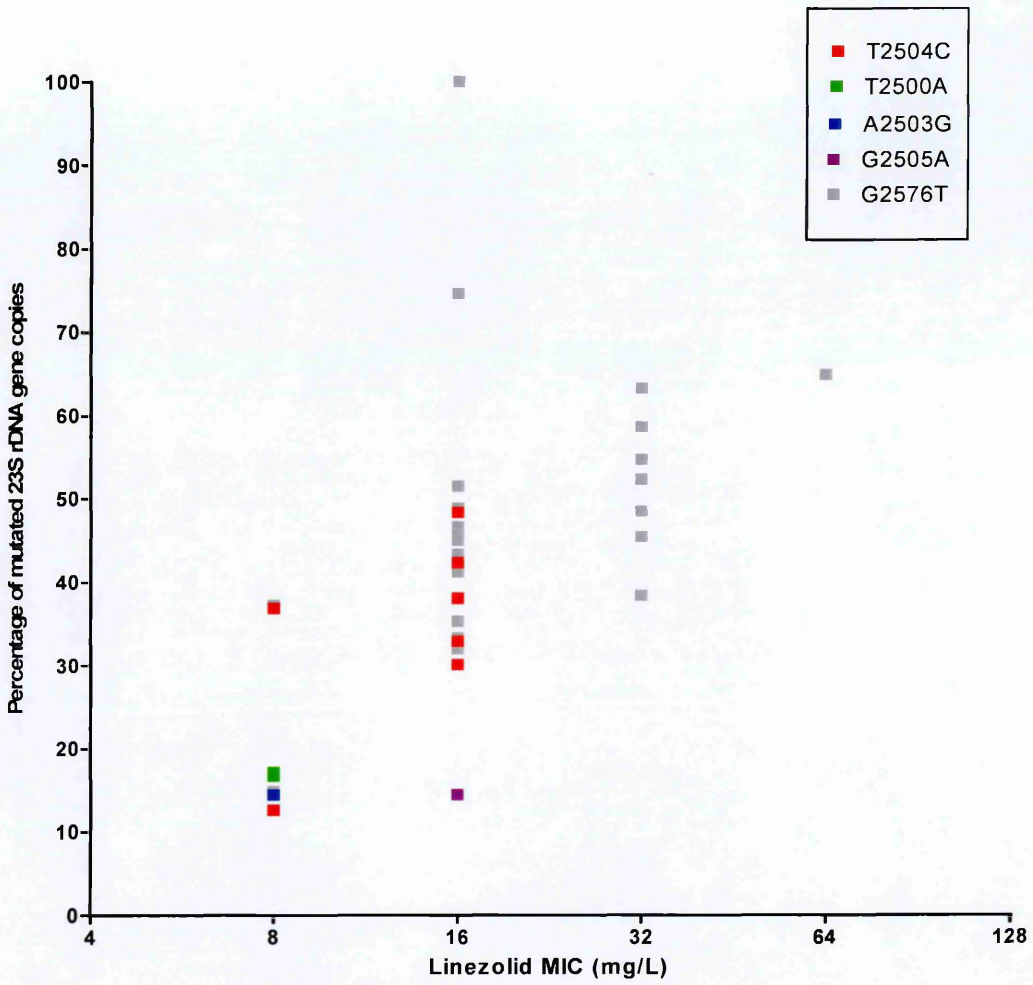


Table 34. Summary of 23S rRNA gene copy number in isolates and mutants tested. (See Table 17, Table 19, Table 26, Table 28, Table 30 and Appendix B Table 55 for individual results).

23S rRNA copy no.	Controls	Laboratory mutants		Clinical isolates		Total
		Lin^S	Lin^R	Lin^S	Lin^R	
4		1	1	1	3	**6**
5	8	11	7	1	3	**30**
6	3	21	31	_	3	**58**
7	_	_	4	_	_	**4**
8	_	1	2	_	_	**3**
9	_	1	1	_	_	**2**
Total	**11**	**35**	**46**	**2**	**9**	**103**

changes in 23S rRNA gene copy number when compared with parent strains, with the majority of these having one extra copy (Table 35). Clinical isolates 1b and 2b had one 23S rRNA gene copy fewer than their precursors. There were no differences in 23S rRNA gene copy number between other sets of clinical isolates from individual patients.

3.6.3 Determination of the number of mutated 23S rRNA gene copies

Hybridization and pyrosequencing were used in conjunction to determine the number of mutated 23S rRNA gene copies in all linezolid-resistant mutants and isolates. The number of mutated 23S rRNA copies ranged from one to four (Table 36). Of 17 laboratory-selected mutants with G2576T mutation, most (seven) had three gene copies mutated. Of nine linezolid-resistant clinical isolates, all with G2576T mutations, four had three gene copies mutated, with the number of mutant copies ranging from two to four. Of seven mutants with T2504C mutations, equal numbers of mutants had two and three copies mutated. Mutants with T2500A, A2503G and G2505A mutations had one copy mutated. There was a positive correlation between linezolid MIC and the number of mutated 23S rRNA gene copies (Figure 57). Overall, the data showed the majority of mutants to have a G2576T mutation, a linezolid MIC of 16 mg/L and three 23S rRNA gene copies mutated.

3.6.4 Summary

Pyrosequencing successfully identified six of seven known 23S rRNA mutations conferring linezolid resistance and allowed five to be quantified. There was a positive correlation between the proportion of mutated copies and the linezolid MIC. The majority of isolates and mutants had 40-50% of 23S rRNA copies mutated. Gene copy number in control strains varied, with the majority having five copies. 23S rRNA gene copy number in mutants exposed to linezolid and chloramphenicol varied, the majority having six. 23S rRNA gene copy number varied between parent and mutant strains. The majority of laboratory-selected linezolid-resistant mutants had G2576T mutations (17/27), three gene copies mutated (6/17) and a linezolid MIC of 16 mg/L

Table 35. Summary of changes in 23S rRNA gene copy number between parents and laboratory-selected mutants. (See Table 17, Table 19, Table 26, Table 28, Table 30 and Appendix B, Table 55 for individual results)

Changes in 23S rRNA copy no.	Lin-susceptible	Lin-resistant	All laboratory mutants
- 1	2	4	6
0	17	25	42
+ 1	14	10	24
+ 2	0	5	5
+ 3	1	1	2
+ 4	1	1	2

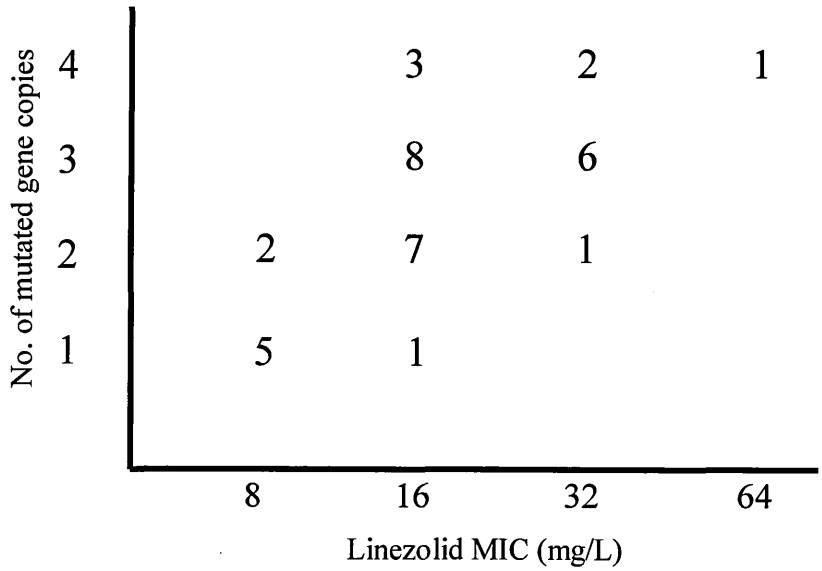
Table 36. Summary of pyrosequencing and hybridization results to identify the number of mutated 23S rRNA gene copies in linezolid-resistant isolates and mutants. (See Table 17, Table 19, Table 26, Table 28, Table 30 and Appendix B Table 55 for individual results)

	G2576T		T2504C	T2500A	A2503G	G2505A	
No. 23S rRNA gene copies mutated	**Clinical**	**Lab**	**All**	**All**	**All**	**All**	**Total**
1	0	1	1	2	1	1	**6**
2	3	4	3	0	1[B]	0	**11**
3	4	7	3	0	0	0	**14**
4	2	5[B]	0	0	0	0	**7**
Total	**9**	**17**	**7**	**2**	**2**	**1**	**38**[A]

[A] Totals do not include 13 mutants with G2447T mutations.

[B] Data includes mutant RN4220Δ*mutS*-M9a which had two mutations present, G2576T and A2503G

Figure 57. Number of 23S rRNA mutated gene copies versus linezolid MIC (mg/L). Numbers indicate isolates with each combination. Mutant RN4220Δ*mutS*-M9a has not been included in this graph due to the presence of two mutations.



(12/27). The majority of linezolid-resistant clinical isolates had G2576T mutations (9/9), 3 gene copies mutated (4/9) and a linezolid MIC of 16 mg/L (5/9).

3.7 Teicoplanin-resistant clinical isolates

Clinical *S. aureus* isolates, resistant to teicoplanin and sent to ARMRL for testing (2003 to 2005), were collected. As with linezolid it was hypothesised that isolates such as these might be hypermutable and, having already undergone chromosomal mutations to become teicoplanin-resistant, might be predisposed to generating linezolid resistance more readily. The frequency at which these isolates generated mutants to rifampicin and fusidic acid was therefore determined. Additionally, fosfomycin disc tests were used to screen for hypermutability in general.

3.7.1 Mutation frequencies

Isolates suspected of being teicoplanin-resistant had MICs determined by agar dilution to determine whether they were above the then breakpoint (≥ 8 mg/L) (see section 2.3.2). The MIC was also determined by E Test (Table 37) (see section 2.3.3). These isolates were further characterized by PFGE (Table 37) (see section 2.6.3). The frequency at which these clinical isolates generated mutants to rifampicin and fusidic acid, individually, at 4 x MIC was determined in three replicated experiments (Table 38) (see section 2.9.1). The mean mutation frequency was calculated (Figure 58 and Figure 59).

Five of 13 teicoplanin-resistant clinical isolates had high-level resistance to rifampicin and therefore no mutation frequency could be established for this drug (Table 38, Figure 58 and Appendix B Table 56). Of the eight teicoplanin-resistant clinical isolates testable, three had mutation frequencies between two- and six-fold higher than RN4220, the remainder had equivalent or lower mutation frequencies. RN4220 mutated to rifampicin at an average mutation frequency of 1.4×10^{-8}. RN4220Δ*mutS* mutated at a frequency 166-fold greater than that of RN4220. RN4220, MSSA (H051000333) and EMRSA-16 (NCTC 13143) isolates all mutated to fusidic acid at similar frequencies. ST/03/2121 and EMRSA-15 (NCTC 14132) had mutation frequencies two- and five-fold higher than RN4220.

Table 37. MICs (mg/L) for teicoplanin-resistant clinical isolates.

					MICs (mg/L							
Patient No.	**Place of isolation**	**Site of isolation**	**Isolate**	**Epidemic strain**	**Teic**	**Lin**	**Oxa**	**Vanc**	**Rif**	**Rif (4 x MIC)**	**Fus**	**Fus (4 x MIC)**
6	Surrey	Skin infection	H034840069	EMRSA-15	16	2	>16	4	0.016	0.064	2	8
7	London	Blood infection	H035220342	EMRSA-16	8	2	>16	4	0.016	0.064	0.064	0.25
8	Surrey	Not provided	H041340156	EMRSA-16	16	2	>16	2	0.016	0.064	1	4
9	Hampshire	Skin infection	H041560345	EMRSA-15	16	1	>16	4	0.032	0.125	16	64
10	Hampshire	Skin infection	H041560346	EMRSA-15	16	2	>16	2	>256	N/A	0.125	0.5
11	Hampshire	Skin infection	H041560348	EMRSA-15	16	2	>16	2	0.008	0.032	8	32
12	Gloucestershire	Septicaemia	H042240308	EMRSA-15	8	1	>16	2	0.016	0.064	>256	N/A
13	London	Chest infection	H043100413	EMRSA-16	8	1	>16	2	0.008	0.032	0.5	2
14	London	Blood infection	H043740188	EMRSA-16	8	2	>16	1	0.008	0.032	0.25	1
15	Middlesex	Skin infection	H044640520	EMRSA-16	8	4	>16	2	>256	N/A	0.25	1
16	Middlesex	Sputum	H044640521	EMRSA-16	8	2	>16	2	>256	N/A	0.125	0.5
17	Middlesex	Drain site	H044920446	EMRSA-16	8	2	>16	2	>256	N/A	0.125	0.5
18	Middlesex	Wound site	H045000304	EMRSA-16	8	2	>16	2	>256	N/A	0.125	0.5

Table 38. Summary of mutation frequencies of teicoplanin-resistant isolates to rifampicin and fusidic acid at 4 x MIC. (See Appendix B Table 56 and Table 57 for more detailed results).

		Rifampicin	Fusidic acid
Patient no.	**Isolate**	**Av. mutation freq and SD**	**Av. mutation freq and SD**
N/A	RN4220	$1.44 \pm 0.26 \times 10^{-8}$	$5.04 \pm 1.83 \times 10^{-8}$
N/A	RN4220Δ*mutS*	$2.39 \pm 2.14 \times 10^{-6}$	$1.55 \pm 0.39 \times 10^{-6}$
N/A	ST/03/2121	$2.24 \pm 0.59 \times 10^{-8}$	$3.29 \pm 1.98 \times 10^{-7}$
N/A	ST/03/2122	N/A[A]	$2.26 \pm 0.77 \times 10^{-7}$
N/A	H051000333 (MSSA)	$1.35 \pm 0.33 \times 10^{-8}$	$1.42 \pm 0.46 \times 10^{-8}$
N/A	NCTC 13142 (EMRSA-15)	$6.80 \pm 5.21 \times 10^{-8}$	$<4.01 \times 10^{-10}$ [B]
N/A	NCTC 13143 (EMRSA-16)	$1.30 \pm 0.67 \times 10^{-8}$	$3.87 \pm 0.72 \times 10^{-8}$
6	H034840069	$8.62 \pm 3.41 \times 10^{-8}$	$5.95 \pm 1.94 \times 10^{-7}$
7	H035220342	$1.83 \pm 0.64 \times 10^{-8}$	$3.24 \pm 0.91 \times 10^{-8}$
8	H041340156	$2.11 \pm 1.20 \times 10^{-8}$	$8.76 \pm 4.86 \times 10^{-9}$
9	H041560345	$4.26 \pm 2.11 \times 10^{-8}$	5.00×10^{-10} [C]
10	H041560346	N/A[A]	$3.38 \pm 2.07 \times 10^{-8}$
11	H041560348	$2.98 \pm 0.74 \times 10^{-8}$	$9.76 \pm 2.68 \times 10^{-10}$

Cont.

Table 38. Summary of mutation frequencies of teicoplanin-resistant isolates to rifampicin and fusidic acid at 4 x MIC (cont.)

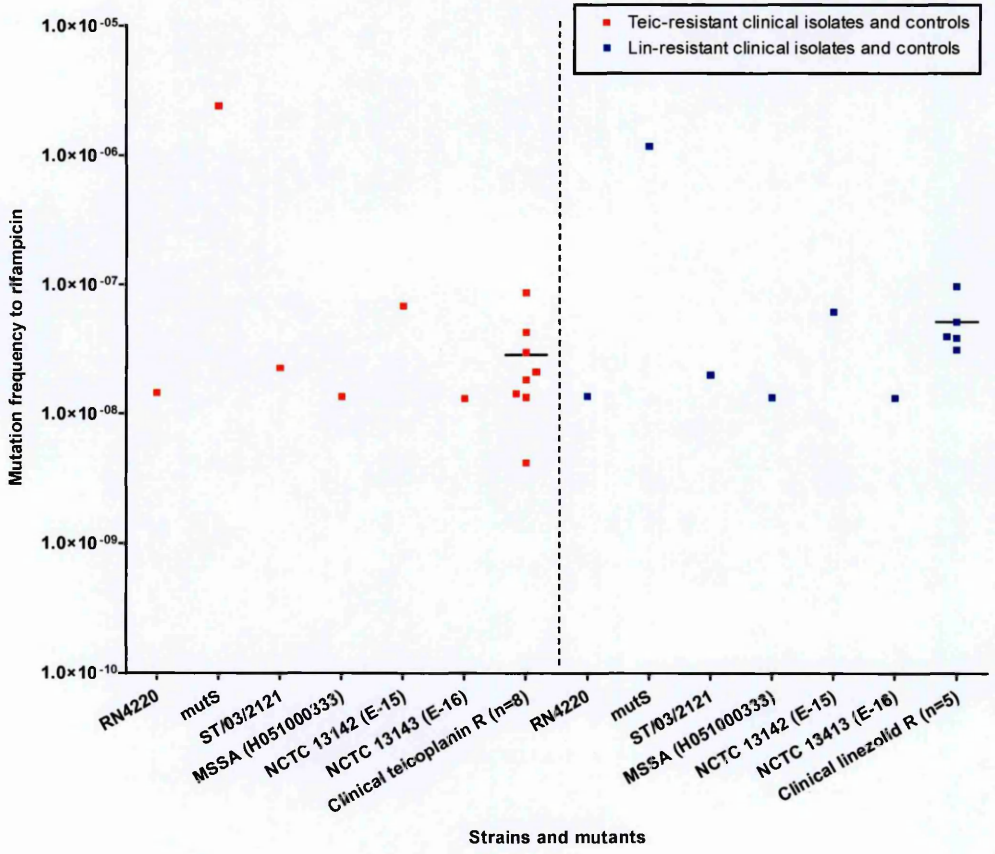
		Rifampicin	Fusidic acid
Patient no.	**Isolate**	**Av. mutation freq and SD**	**Av. mutation freq and SD**
12	H042240308	$4.17 \pm 2.93 \times 10^{-9}$	N/A[A]
13	H043100413	$1.43 \pm 0.50 \times 10^{-8}$	$2.44 \pm 1.55 \times 10^{-9}$
14	H043740188	$1.34 \pm 0.46 \times 10^{-8}$	$4.22 \pm 1.66 \times 10^{-9}$
15	H044640520	N/A[A]	$2.08 \pm 0.04 \times 10^{-9}$
16	H044640521	N/A[A]	$5.22 \pm 2.15 \times 10^{-9}$
17	H044920446	N/A[A]	$7.33 \pm 0.64 \times 10^{-9}$
18	H045000304	N/A[A]	$3.04 \pm 1.16 \times 10^{-9}$

[A] A mutation frequency was not calculable due to high-level rifampicin resistance.

[B] Mutation frequency was below the detectable limit.

[C] Only one mutation frequency was measurable from the three replicated experiments, results from the remainder were below the detection limit.

Figure 58. Mutation frequencies to rifampicin for teicoplanin-resistant isolates. Each dot represents an arithmetic mean mutation frequency of three replicated experiments. Bars represent the mean mutation frequency for a group of isolates.



Mutation frequencies to fusidic acid were determined for all but one of the 13 isolates (Table 38, Figure 59 and Appendix B, Table 57). RN4220 mutated at a frequency of 5.0×10^{-8}. RN4220Δ*mutS* mutated to fusidic acid at a frequency 31-fold higher than RN4220. Isolates ST/03/2121, ST/03/2122 and the control strain EMRSA-16 mutated at seven-, four- and three-fold greater than RN4220, respectively. The MSSA control strain and isolates H035220342 and H041560346 mutated at the same frequency as RN4220. Isolate H034840069 was the only isolate to produce a mutation frequency to fusidic acid greater than RN4220 (12-fold increase). Eight isolates mutated at a frequency six to 100-fold lower than RN4220. The control strain EMRSA-15 and H042240308 mutated at a frequency to fusidic acid below the detectable limit. The mutation frequency for isolate H041560345 was 100-fold lower than RN4220 and was only detected from one experiment. The other two experiments had mutation frequencies below the detectable limit.

One isolate, H034840069, had mutation frequencies elevated above those produced by RN4220 to both rifampicin and fusidic acid; a six-fold increase to rifampicin and a 12 fold-increase to fusidic acid. However, ST/03/2121 also produced mutation frequencies two-fold and seven-fold greater than RN4220 to rifampicin and fusidic acid, respectively.

Figure 58 and Figure 59 compare the mutation frequencies to rifampicin and fusidic acid of teicoplanin-resistant clinical isolates with linezolid-resistant clinical isolates. There are no obvious differences in the mutation frequencies of the control strains between the two data sets. Linezolid-resistant clinical isolates, on average, mutated at a greater frequency to rifampicin (two-fold) and fusidic acid (8-fold) than teicoplanin-resistant clinical isolates.

3.7.2 Fosfomycin disc tests

The number of colonies in the zones of inhibition around 50μg fosfomycin discs were counted in three replicated experiments and the diameter of zones was also measured (Table 39) (see section 2.9.2). The arithmetic mean of these measurements was calculated and graphed (Figure 60 and Figure 61). Wild-type control strains MSSA, EMRSA-15 and EMRSA-16 produced, on average, between 42 and 76 more colonies in the zone of clearing around a fosfomycin disc than RN4220. RN4220Δ*mutS* produced, on average, 191 more colonies than RN4220. Two isolates had four and

Figure 59. Mutation frequency to fusidic acid for teicoplanin-resistant isolates. Each dot represents an arithmetic mean mutation frequency of 3 replicated experiments. Bars represent the mean mutation frequency for a group of isolates.

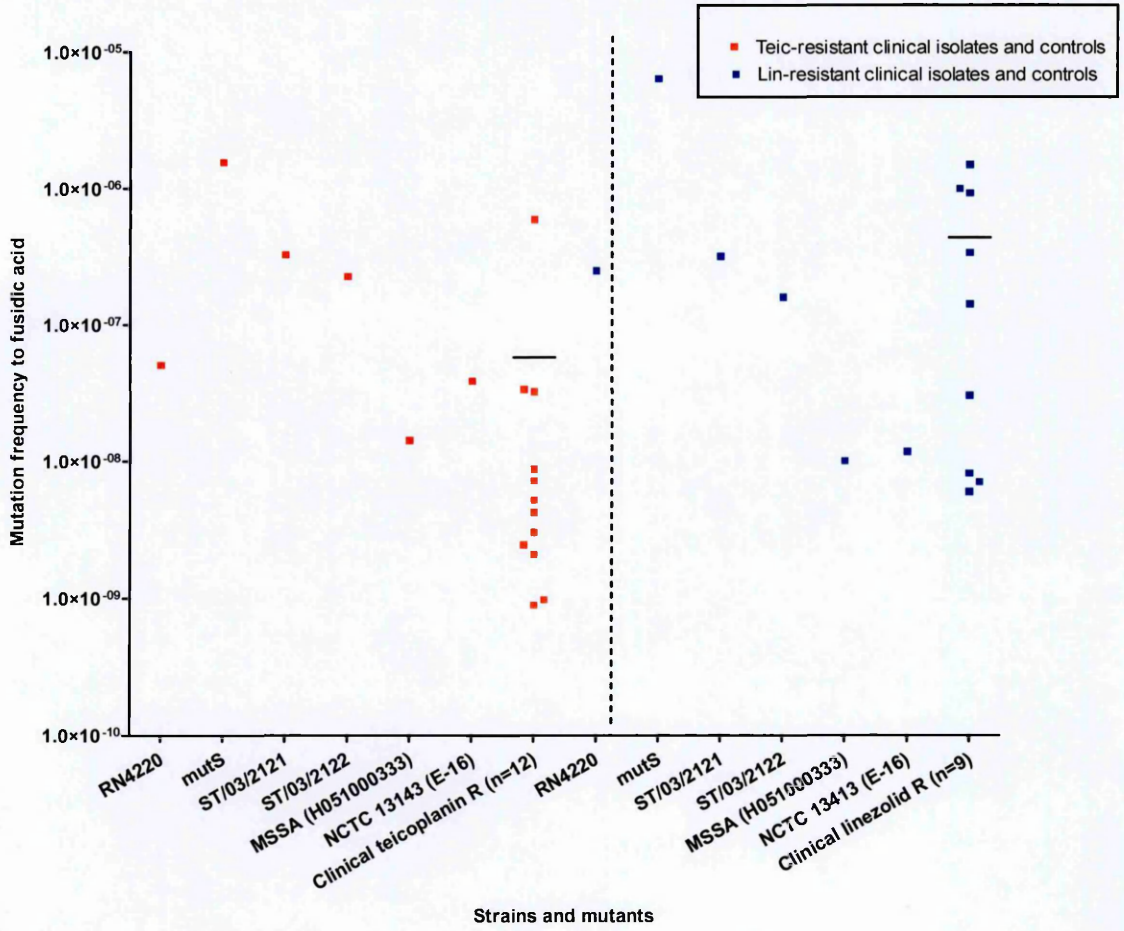


Table 39. Number of colonies in the zone of clearing and zone diameter produced by teicoplanin-resistant clinical isolates around fosfomycin discs (50 µg).

Patient No.	Strain	Teic MIC (mg/L)	No. of colonies in zone			Average ± SD	Zone diameter (mm)			Average ± SD
N/A	RN4220	1	66	53	59	**60 ± 9**	41.8	42.0	43.7	**42.5 ± 0**
N/A	RN4220Δ*mutS*	2	302	267	306	**292 ± 25**	43.8	43.8	44.3	**43.9 ± 0**
N/A	ST/03/2121	1	276	243	234	**251 ± 23**	42.4	41.3	42.4	**42.0 ± 1**
N/A	ST/03/2122	8	128	148	183	**153 ± 14**	39.3	40.2	39.4	**39.6 ± 1**
N/A	MSSA	2	133	102	127	**121 ± 22**	37.8	36.6	35.0	**36.5 ± 1**
N/A	EMRSA-15	1	178	123	108	**136 ± 39**	32.3	32.0	31.8	**32.0 ± 0**
N/A	EMRSA-16	2	103	110	94	**102 ± 5**	33.1	30.9	31.9	**32.0 ± 2**
6	H034840069	16	244	245	240	**243 ± 1**	43.9	40.8	45.4	**43.4 ± 2**
7	H035220342	8	306	276	283	**288 ± 21**	33.7	30.9	33.3	**32.6 ± 2**
8	H041340156	16	126	112	101	**113 ± 10**	29.6	31.4	29.7	**30.3 ± 1**
9	H041560345	16	38	81	48	**56 ± 30**	29.9	30.2	30.3	**30.1 ± 0**
10	H041560346	16	131	144	102	**126 ± 9**	33.2	35.0	36.2	**34.8 ± 1**
11	H041560348	16	164	259	141	**188 ± 67**	29.3	29.9	28.4	**29.2 ± 0**
12	H042240308	8	51	59	76	**62 ± 6**	56.1	55.9	55.7	**55.9 ± 0**
13	H043100413	8	34	39	36	**36 ± 4**	26.7	25.0	26.3	**26.0 ± 1**

Cont.

Table 39. Number of colonies in the zone of clearing and zone diameter produced by teicoplanin-resistant clinical isolates around fosfomycin discs (50μg) (cont.).

Patient No.	Strain	Teic MIC (mg/L)	No. of colonies in zone			Average ± SD	Zone diameter (mm)			Average ± SD
14	H043740188	8	113	140	106	**120 ± 19**	35.3	33.7	34.7	**34.6 ± 1**
15	H044640520	8	135	120	149	**135 ± 11**	35.8	34.9	35.8	**35.5 ± 1**
16	H044640521	8	123	178	174	**158 ± 39**	33.2	36.6	33.7	**34.5 ± 2**
17	H044920446	8	59	56	93	**69 ± 2**	35.4	34.8	36.3	**35.5 ± 0**
18	H045000304	8	158	98	143	**133 ± 42**	33.0	36.3	35.9	**35.1 ± 2**

Figure 60. Number of colonies in the zone of inhibition of a fosfomycin discs (50 μg) produced by teicoplanin-resistant clinical isolates. Standard deviations are represented by error bars.

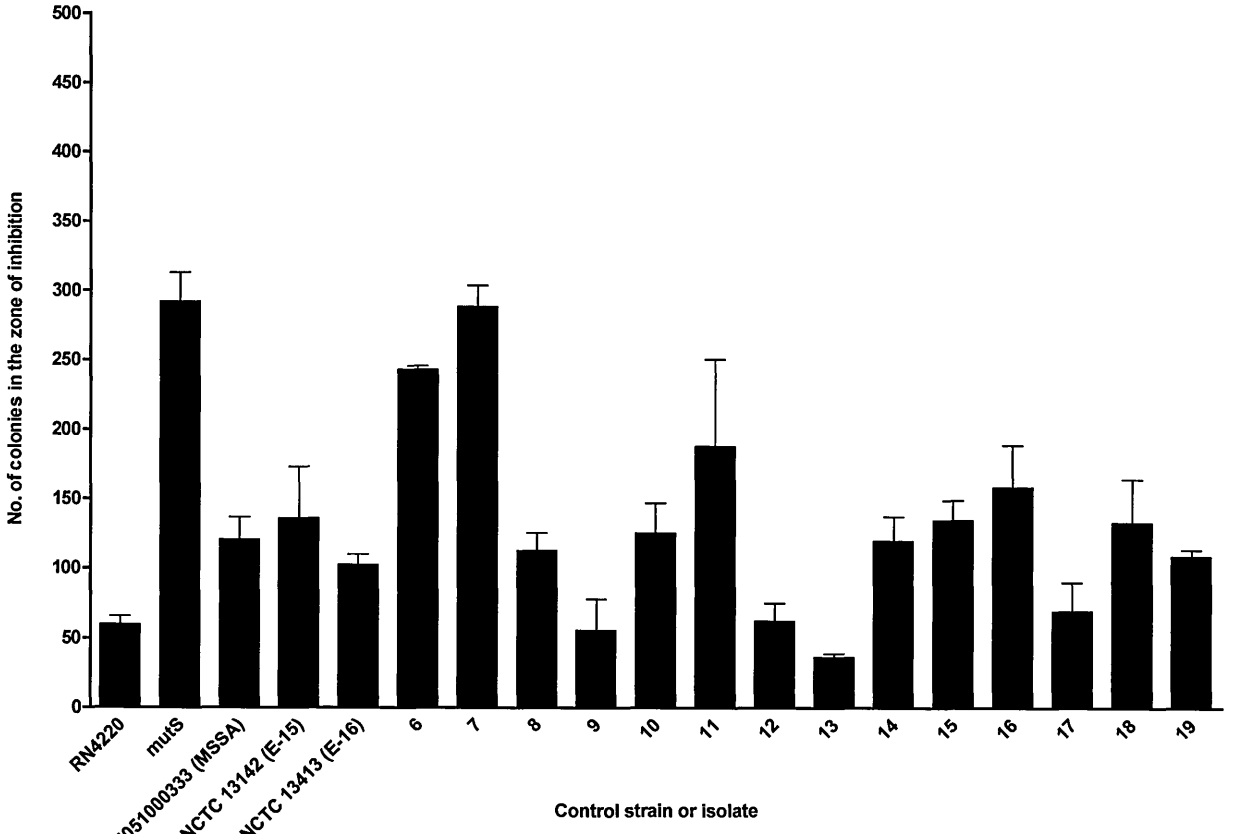
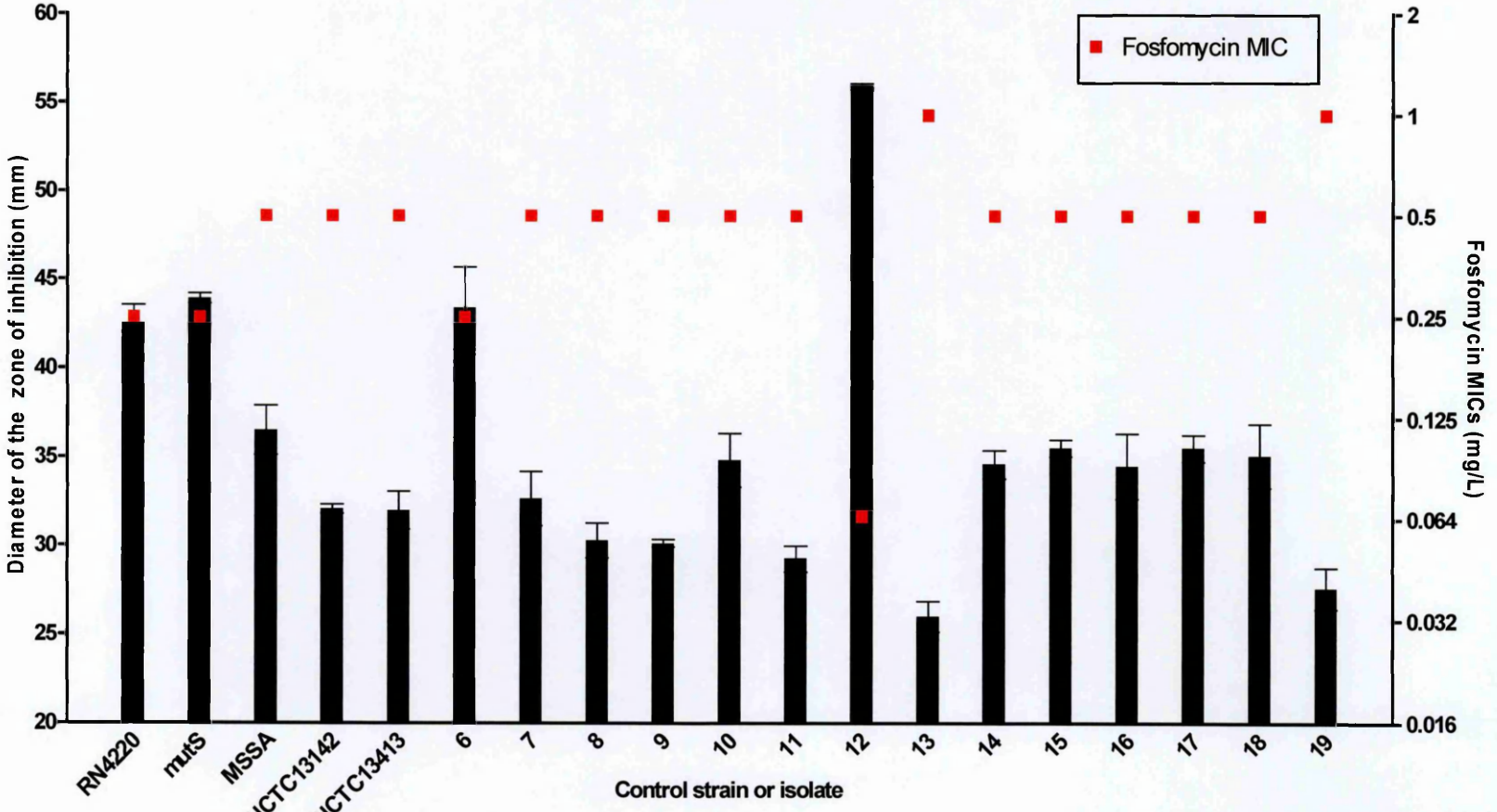


Figure 61. Zone diameter of fosfomycin discs (50 µg) produced by teicoplanin-resistant clinical isolates. Standard deviation is represented by error bars.



24 less colonies in the zone of clearing than RN4220, the remaining ten had between two and 228 more. Four isolates had 22 to 152 more colonies than EMRSA-15. None of these isolates had as many colonies in the zone of clearing as RN4220Δ*mutS*; the nearest isolate produced 64 colonies fewer.

Zone diameters in RN4220 and RN4220Δ*mutS* were similar, 42.5 mm and 43.5 mm, respectively. Only two isolates had bigger zone diameters around a fosfomycin disc than RN4220. Isolate 12 had a zone diameter 13 mm larger than RN4220. Ten isolates had zone sizes between 0.5 mm and 16.5 mm less than RN4220. Only four isolates had zone diameters smaller than EMRSA-15, 1.7 mm to 6 mm smaller. Fosfomycin MICs reflected zone diameters. There is no correlation between zone diameter and the number of colonies in the zone of clearing, nor between fosfomycin MIC and the number of colonies in the zone of clearing.

3.7.3 Summary

Only one clinical teicoplanin-resistant isolate, H034840069, had mutation frequencies elevated above those for RN4220 to both rifampicin and fusidic acid, however, control isolate ST/03/2121 also produced elevated mutation frequencies. When compared with clinical linezolid-resistant isolates, teicoplanin-resistant isolates had lower mean mutation frequencies to both rifampicin and fusidic acid. Fosfomycin disc tests revealed four isolates that produced more colonies in the zone of inhibition around a fosfomycin disc than the control EMRSA-15 strain. No isolates produced more colonies than RN4220Δ*mutS*. There was no correlation between zone diameter and the number of colonies in the zone of inhibition surrounding a fosfomycin disc.

4 Discussion

4.1 Linezolid resistance in *S. aureus*

The aims of this project were to study the mechanism and development of resistance to linezolid in staphylococci, including assessing the number of mutated 23S rRNA copies in clinical isolates and laboratory-selected mutants, to investigate the role of hypermutability in the emergence of linezolid and teicoplanin resistance and to establish whether teicoplanin resistant isolates express a mutator phenotype.

4.1.1 Mutant generation and characterization

Due to the low number of linezolid-resistant *S. aureus* isolates available from the clinic, linezolid-resistant mutants were selected in the laboratory. This enabled a greater number of linezolid-resistant mutants to be investigated for their mechanisms of resistance and enabled analogies to be made with those from the clinic.

Fifty linezolid-resistant cultures were studied, nine of which were from the clinic, the remainder were laboratory-selected. Additionally, 35 variants in which, after repeated exposure to linezolid, resistance had not emerged or reversion to linezolid susceptibility had occurred were studied. Linezolid MICs ranged from 16-32 mg/L in clinical isolates and 8->256 mg/L in laboratory-selected mutants.

Resistance to linezolid was selected, in the main, by passage in the presence of increasing concentrations of linezolid. This method was successful and enabled the generation of linezolid-resistant mutants, although sometimes this was a lengthy process. Mutations in the 23S rRNA genes conferring resistance to linezolid were detected by PCR-RFLP, sequencing of a 694 bp region of the 23S rRNA genes and pyrosequencing. Additionally, hybridization was used to quantify the number of 23S rRNA gene copies in all mutants exposed to linezolid and their parent strains.

One of the aims of this work was to assess the number of 23S rRNA gene copies that are mutated in linezolid-resistant mutants, both laboratory-selected and from the clinic, as limited work has been carried out on determining, firstly the total number of 23S rRNA genes in linezolid-resistant *S. aureus* isolates and, secondly, the number of these that are mutated (Meka *et al.*, 2004b, Pillai *et*

al., 2002). The number of mutated 23S rRNA gene copies has previously been determined by sequencing five pre-defined published gene copies (Miller *et al.,* 2005, Paterson *et al.,* 2003). However both Meka *et al.*, (2004b) and this work have proved that 23S rRNA gene copy number is variable in isolates of staphylococci exposed to linezolid. Hence just sequencing five published gene copies is not a reliable method for detecting the number of mutated 23S rRNA genes.

This work has developed a new strategy for quantifying the number of mutated 23S rRNA gene copies in linezolid-resistant mutants using a combination of pyrosequencing and hybridization. Pyrosequencing has previously been used to detect and quantify mutations in linezolid-resistant enterococci (Sinclair *et al.,* 2003). Hybridisation has been used previously to determine 23S rRNA gene copy number in *S. aureus* (Meka *et al.,* 2004a, Meka *et al.,* 2004b). Additionally, hybridization after digest with *Nhe*I has been used to detect the number of 23S rRNA gene copies with a G2576T mutation in linezolid-resistant clinical isolates of *S. aureus* (Meka *et al.,* 2004b, Pillai *et al.,* 2002). Pyrosequencing and hybridization have not been used in combination before to enable the detection and quantification of the number of mutated 23S rRNA gene copies in linezolid-resistant isolates or mutants of *S. aureus*.

4.1.2 Linezolid-resistant *S. aureus* clinical isolates

To date there are relatively few published reports of linezolid-resistant *S. aureus* isolates from the clinic (Gales *et al.,* 2006, Hill *et al.,* 2005, Machado *et al.,* 2003, Meka *et al.,* 2004b, Paterson *et al.,* 2003, Pillai *et al.,* 2002, Roberts *et al.,* 2006, Tsiodras *et al.,* 2001, Wilson *et al.,* 2003). In addition, resistant *S. aureus* isolates have been described in a survey (Brauers *et al.,* 2005) and there have been other occurrences that remain unpublished (This work, M. Mulvey personal communication, N. Woodford personal communication). The commonest mutation detected in these isolates from the clinic is a G to T change at position 2576 of the 23S rRNA genes (Gales *et al.,* 2006, Hill *et al.,* 2005, Machado *et al.,* 2003, Paterson *et al.,* 2003, Pillai *et al.,* 2002, Roberts *et al.,* 2006, Tsiodras *et al.,* 2001, Wilson *et al.,* 2003). Heterozygosity and homozygosity at this position has been reported (Gales *et al.,* 2006, Hill *et al.,* 2005, Machado *et al.,* 2003, Pillai *et al.,*

2002, Tsiodras *et al.,* 2001), although reports of homozygous isolates may be incorrect if only five gene copies are sequenced (Paterson *et al.,* 2003, Wilson *et al.,* 2003).

Nine clinical linezolid-resistant *S. aureus* isolates, representing five strains, were studied in this work. They were fully characterized in order to investigate their mechanisms of resistance to linezolid. Consistent with published findings, all of the clinical linezolid-resistant *S. aureus* isolates tested in this study had a G2576T mutation. Two of the nine were confirmed homozygous by multiple methods, the remainder were heterozygous. Linezolid resistance did not seem to develop preferentially in certain strains from the clinic. Resistance emerged in two MSSA isolates and both of the main UK epidemic strains, EMRSA-15 (three isolates) and EMRSA-16 (four isolates). The G2576T mutation was present in 17 laboratory-selected mutants in this study, all of which were heterozygous.

Very few mutants homozygous for the G2576T mutation have been selected *in vitro* (Miller *et al.,* 2005), yet they do occur in the clinic (This work, Pillai *et al.,* 2002, Tsiodras *et al.,* 2001, Wilson *et al.,* 2003). An attempt was made in this study by serially passaging a RN4220Δ*mutS* mutant in the presence of increasing concentrations of linezolid. This did not result in a mutant homozygous for the G2576T mutation, but did produce a mutant with a linezolid MIC of >256 mg/L and two mutations, G2576T and A2503G. Hybridization revealed that this mutant had six 23S rRNA gene copies. From the results of the pyrosequencing it was deduced that the G2576T mutation was present in four gene copies and that the A2503G mutation was present in two gene copies, although it was not determined whether these two mutations were present on different 23S rRNA gene copies, meaning all six were mutated, or whether four copies were mutated with two of the four each harbouring two different mutations. This mutant proved unstable and some reversion towards susceptibility occurred even in the presence of low levels of linezolid, consequently, it had to be grown in the presence of a high concentration of linezolid to maintain a high MIC. Passaging in the presence of very high linezolid concentrations still did not generate a mutant homozygous for the G2576T mutation. Clinical isolates are not exposed to such high levels of linezolid yet homozygosity still occurs. Homozygosity at position 2576 seemed to be more difficult to generate in the laboratory than in the clinic and could be a phenomenon that is strain dependent.

An isolate with two or more different mutations in the 23S rRNA genes, conferring linezolid resistance, has not been isolated from the clinic. However, in the laboratory Miller *et al.*, (2005) have selected linezolid-resistant *recA*-negative mutants of *S. aureus* with up to three different mutations in five copies of the 23S rRNA genes (G2576T, G2447T and T2500A). It is not clear whether the mutations were in the same or different 23S rRNA gene copies and there was no mention of the stability of these mutants.

It is not yet apparent why the G2576T mutation is so dominant amongst linezolid-resistant isolates of both enterococci and staphylococci from the clinic. The growth rates of clinical linezolid-resistant isolates of *S. aureus* have been compared to wild-type strains in order to determine whether there is a fitness cost associated with this mutation. Pillai *et al.*, (2002) found a linezolid-resistant *S. aureus* isolate, with a G2476T mutation, had *in vitro* growth at a rate similar to linezolid-susceptible strains and Gales *et al.*, (2006) found a *S. aureus* clinical isolate with a G2576T mutation did not show any significant decrease in growth *in vitro*. Similarly, Meka *et al.*, (2004b) saw no appreciable difference in growth rates between susceptible and linezolid-resistant *S. aureus* strains with a G2576T mutation in the 23S rRNA genes, although a difference in colony morphology was noted. Contrary to this, Lobritz *et al.*, (2003) found an inverse relationship between the number of mutated 23S rRNA genes (G2576T) and growth rate in linezolid-resistant mutants of *E. faecalis*.

If the G2576T mutation carries no fitness cost, or less of a fitness cost than other mutations, it would be expected to be selected over other mutations conferring resistance and therefore be the most successful mutation. This does not explain why it is possible to generate other mutations in the laboratory, although *in vitro* conditions do not mimic those *in vivo* resulting in the selection of different mutations. Studies are needed to compare the fitness costs of the different mutations to see whether the G2576T mutation has a fitness cost associated with it, or less of a fitness cost than other mutations.

Only one other mutation, a T to A change at position 2500, has been identified from a linezolid-resistant *S. aureus* isolate from the clinic (Meka *et al.*, 2004b). Similarly, in enterococci from the clinic no mutations other than G2576T have been detected. It has been possible to select the G2576T and T2500A mutations, associated with isolates from the clinic, in the laboratory (Barrett,

2000, Bryskier, 1999, Howe *et al.,* 2002, Miller *et al.,* 2005, North *et al.,* 2005a, North *et al.,* 2005b, Swaney *et al.,* 1998a). The T2500A mutation was not identified in any of the linezolid-resistant clinical isolates tested in this study, but was identified in two laboratory-selected mutants (see sections 3.3, 3.4 and 4.1.7). Other mutations were also detected in laboratory-selected linezolid-resistant mutants including a G to T change at position 2447, which has previously been detected in enterococci and staphylococci (Miller *et al.,* 2005, Swaney *et al.,* 1998a, Wichelhaus *et al.,* 2003). Thirteen mutants with this mutation were selected in this study; all were heterozygous. Interestingly, another laboratory-selected mutation previously detected in staphylococci, a G to T change at position 2445, was not detected in this study (Barrett, 2000). Mutations G2505A, A2503G and T2504C were also identified in laboratory-selected linezolid-resistant mutants (see section 4.1.7).

4.1.3 Alternative mechanisms of linezolid resistance

Five laboratory-selected linezolid-resistant mutants had no detectable mutations in a 694 bp sequenced region of the 23S rRNA genes (MICs 8-16 mg/L), furthermore, other mutants only had one 23S rRNA gene copy mutated, which is conventionally thought to be insufficient to confer resistance. This implies that there is another mechanism or mechanisms of linezolid resistance or that there are mutations outside of domain V of the 23S rRNA genes that can confer resistance. This is a finding previously encountered by others. Clinical isolates of staphylococci, have been isolated with no detectable mutations in the 23S rRNA genes (M.Mulvey personal communication, Fraimow *et al.,* 2005). Likewise, Carsenti-Dellamonica *et al.,* (2005) could not detect any mutations in the 23S rRNA genes of linezolid-resistant mutants of streptococci selected *in vitro.* Rare linezolid-resistant isolates of enterococci from the clinic and laboratory-selected have had no detectable mutations (N. Woodford, personal communication, Prystowsky *et al.,* (2001)).

These examples indicate that there are alternative mechanisms, besides mutations in the 23S rRNA genes, which encode for resistance to linezolid. One possible mechanism is mutations in the ribosomal protein L4. Mutations in the L4 ribosomal protein, with and without the addition of mutations in the 23S rRNA genes, have been encountered in pneumococci and streptococci (Farrell

et al., 2004, Wolter *et al.*, 2005). Furthermore, it was suggested linezolid may bind to proteins L27 and Lep and mutations in these genes may confer a degree of resistance (Colca *et al.*, 2003). There are, as yet, no reports of mutations in ribosomal proteins conferring resistance to linezolid in enterococci or staphylococci, either *in vivo* or *in vitro*.

4.1.4 Proliferation of linezolid resistance

It was originally thought that independent, spontaneous mutations would have to occur in multiple copies of the 23S rRNA genes to enable phenotypic linezolid resistance to occur; a feat that was proposed to be virtually impossible (Xiong *et al.*, 2000). However, resistance to linezolid did occur in the clinical and laboratory, indicating resistance was occurring via another route. Lobritz *et al.*, (2003) proposed that proliferation of mutations within an individual mutant occurred as a result of homologous recombination between resistant and susceptible 23S rRNA genes, i.e., a spontaneous mutation in one 23S rRNA gene copy is transferred to at least one other resulting in phenotypic resistance. Lobritz *et al.*, (2003) provided evidence to support this theory by demonstrating *recA*-negative mutants of enterococci, upon exposure to linezolid, were only able to generate a (G2505A) mutation in one 23S rRNA gene copy. In comparison, *recA*-positive mutants exposed to linezolid had (G2576T) mutations in a minimum of two copies. Similarly, Miller *et al.*, found *recA*-negative mutants of *S. aureus* generated three different mutations in five 23S rRNA gene copies when grown in the presence of linezolid. Conversely, *recA*-positive strains had identical mutations (G2576T) in five gene copies. This proved that in *recA*-negative strains, without homologous recombination, proliferation of a single mutation throughout the 23S rRNA gene copies does not occur. It seems that in an attempt to generate linezolid resistance, different types of mutations occur spontaneously in different gene copies. This indicates that linezolid resistance could occur without homologous recombination, however, this is statistically improbable. Lobritz *et al.*, (2003) and Miller *et al.*, (2005) both concluded that homologous recombination is the mechanism by which the proliferation of linezolid resistance occurs in clinical isolates. Lobritz *et al.*, (2003) also stated that the emergence of the first mutation in the 23S rRNA genes is the rate limiting step for this process.

If recombination is the mechanism by which a mutation is transferred to other 23S rRNA gene copies, it is probably the route by which reversion to susceptibility occurs. However, in order to revert to linezolid susceptibility one wild-type 23S rRNA copy needs to remain in order to act as a non-mutated template (Meka *et al.,* 2004a). Isolates with all copies mutated would not be able to revert to susceptibility unless a wild type 23S rRNA template was obtained from an exogenous source or via a mutational event. Consistent with this theory Pillai *et al.*, (2002) found a linezolid-resistant *S. aureus* mutant, homozygous for the G2576T mutation, remained unchanged after 15 passages in the absence of linezolid. Similarly, clinical linezolid-resistant isolates from this study, homozygous for the G2576T mutation, proved stable with no reversion towards susceptibility occurring in the absence of linezolid.

4.1.5 Stability of linezolid resistance

Whilst selecting linezolid-resistant mutants it was noted that heterozygous linezolid resistance was more stable in isolates from the clinic whereas those selected in the laboratory tended to revert to susceptibility if passaged repeatedly on antibiotic-free agar. Other published reports of unstable linezolid resistance have been published. Meka *et al.*, (2004a) reported a clinical isolate in which some reversion had occurred after 30 passages and complete reversion had occurred after 60 passages on antibiotic-free agar. Kaatz *et al.*, (1996) found laboratory-selected mutants of staphylococci were only stable for three passages in the absence of linezolid.

It is possible that a specific, preferential 23S rRNA gene copy needs to be mutated in order for resistance to become stable; if this gene copy is not mutated, unstable resistance occurs. It has been shown that in the clinic, in patients where linezolid resistance has occurred, the removal of linezolid therapy led to the isolation of bacteria susceptible to linezolid, yet still with one 23S rRNA gene copy mutated. Subsequent re-introduction of treatment with linezolid has led to the proliferation of resistance among the remaining 23S rRNA gene copies and consequently resulted in phenotypic resistance (Geiss *et al.,* 2005). It is possible that the one copy that remained mutated could be the stabilising copy, which, enabled rapid proliferation amongst the remaining wild-type 23S rRNA gene copies upon re-exposure to linezolid.

4.1.6 Gene dosage effect

It has been shown that there is a positive correlation between the number of mutated 23S rRNA gene copies and linezolid MIC for enterococci (Marshall *et al.*, 2002). It has been proposed that a similar relationship exists in staphylococci (Meka *et al.*, 2004a, Swaney *et al.*, 1998a, Wilson *et al.*, 2003, Wichelhaus *et al.*, 2003). One of the aims of this work was to investigate whether the gene dosage effect also exists in *S. aureus* linezolid-resistant mutants from the clinic and those selected *in vitro*. After fully characterising all the linezolid-resistant mutants by pyrosequencing and hybridisation it was concluded that there is a positive correlation between the number of mutated 23S rRNA gene copies and the linezolid MIC. The pyrosequencing data alone proved there was a positive correlation between the percentage of mutated 23S rRNA gene copies and the linezolid MIC. Additionally, the pyrosequencing and hybridization data combined proved there was a positive correlation between the actual number of mutated 23S rRNA genes and the linezolid MIC in clinical and laboratory-selected mutants of *S. aureus*. The pyrosequencing method had one drawback; detection of a G2447T mutation was possible, however, due to this mutation residing within a homopolymer of four G nucleotides, limitations of the proprietary software meant quantification of the number of wild-type and mutated 23S rRNA gene copies was not feasible. For mutations other than G2576T, a gene dosage effect was harder to determine due to the low numbers involved or the inability to determine the number of 23S rRNA gene copies in the case of G2447T mutations.

Analysis of the linezolid-resistant clinical *S. aureus* isolates using pyrosequencing and hybridization revealed 36% to 100 % of 23S rRNA gene copies were mutated, while hybridization revealed that four to six gene copies were present. By combining these results it was determined that two to four 23S rRNA gene copies were mutated (linezolid MICs 16-32 mg/L). In laboratory-selected mutants, with a G2576T mutation, the percentage of mutated 23S rRNA gene copies ranged from 15% to 65% and the number of mutated 23S rRNA gene copies ranged between one and four.

A comparison of the linezolid-resistant isolates from the clinic and those selected in the laboratory revealed linezolid-resistant isolates from the clinic had a minimum of two 23S rRNA gene copies mutated, whereas phenotypic linezolid resistance was conferred by even one mutated copy in those

mutants selected *in vitro*. From the literature a similar picture emerges with isolates from the clinic usually having a minimum of two and a maximum of five gene copies mutated (Paterson *et al.*, 2003, Pillai *et al.*, 2002, Tsiodras *et al.*, 2001, Wilson *et al.*, 2003).

A linezolid-resistant *S. aureus* isolate from the clinic, reportedly homozygous for the G2576T mutation, with a linezolid MIC of 64 mg/L and all five sequenced gene copies mutated has previously been reported (Pillai *et al.*, 2002). Interestingly, in this study, two clinical isolates (linezolid MICs 16 mg/L) from the same patient had G2576T mutations in all four gene copies. In functional terms, the homozygous isolates reported here and that reported by Pillai *et al.*, (2002) should have similar linezolid MICs as four of four mutated 23S rRNA gene copies should be equivalent to five of five mutated gene copies as in both cases the target site has been altered and there are no wild-type copies available, but this does not seem to be the case.

Hybridization revealed that the control and parent strains used in this study had five or six 23S rRNA gene copies, indicating five 23S rRNA gene copies is not always the norm. Furthermore, the linezolid-resistant clinical isolates tested had four, five or six 23S rRNA gene copies. After exposure to linezolid, changes in 23S rRNA copy number occurred in 46% of mutants exposed to linezolid, with the majority increasing in gene copy number by one. Changes in 23S rRNA copy number have not previously been associated with the G2576T mutation in staphylococci, but loss of a gene copy was reported in a clinical linezolid-resistant *S. aureus* isolate with a T2500A mutation (Meka *et al.*, 2004b). Meka *et al.*, (2004b) proposed that loss of a 23S rRNA gene copy would increase the overall proportion of mutant copies relative to wild-type copies. The findings here are inconsistent with this theory. It is possible that increasing the number of functional, non-mutated 23S rRNA gene copies allows greater flexibility and incurs less of a fitness cost. Not all mutants had altered 23S rRNA gene copy number but some did have differences in the hybridisation RFLP banding profiles. For three laboratory-selected mutants, although 23S rRNA gene copy remained the same as their parent strains, the banding profiles changed indicating selected detectable mutations occurred in the enzyme recognition sequence downstream of the 23S rRNA genes themselves.

The evidence from this study indicates that 23S rRNA gene copy number is variable in linezolid-resistant mutants and in those mutants which are exposed to linezolid but in which resistance does

not emerge. Therefore the assumption that all clinical linezolid-resistant and laboratory-selected isolates have five 23S rRNA gene copies (Miller *et al.,* 2005, Paterson *et al.,* 2003, Wilson *et al.,* 2003) may be misleading. For the full characterization of linezolid-resistant mutants the 23S rRNA gene copy number needs to be established before the number of mutated gene copies can be determined.

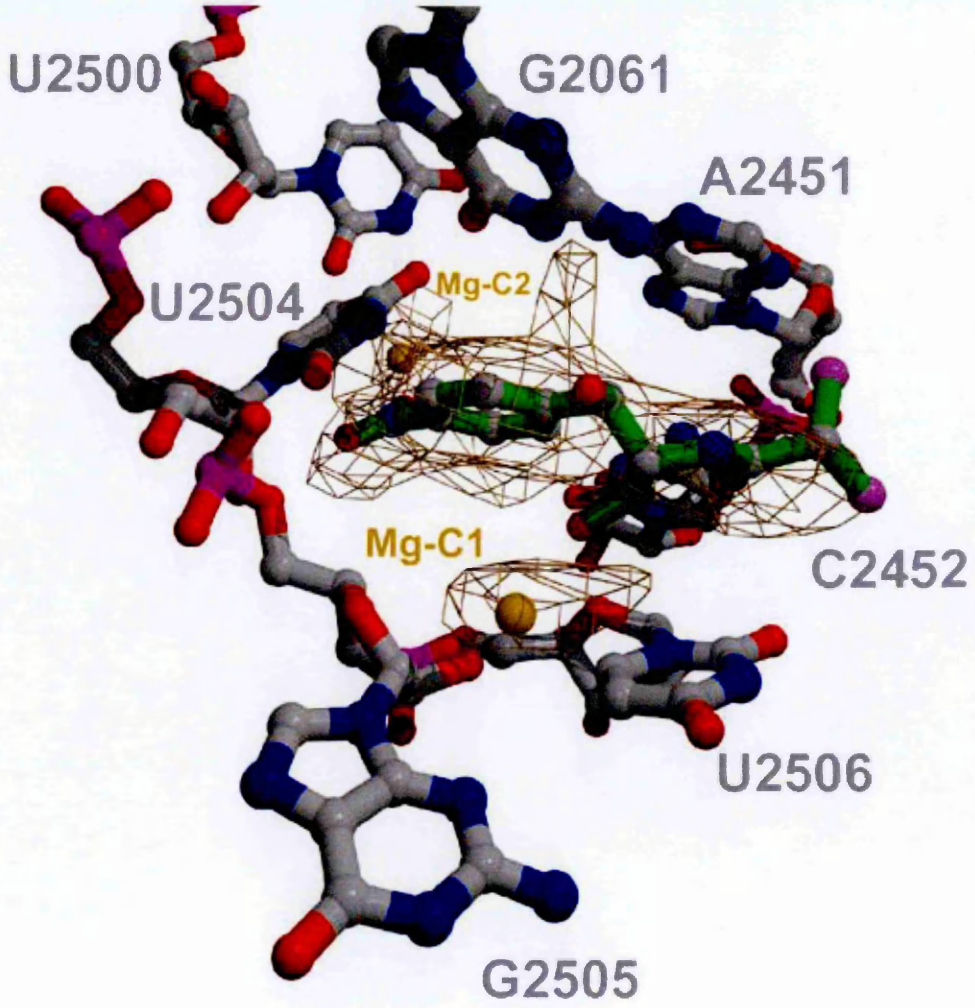
4.1.7 Cross-resistance between linezolid and other classes of antibiotics

Cross-resistance between linezolid and other protein synthesis inhibitors is generally thought not to occur. However, whilst generating linezolid-resistant mutants in the laboratory it was discovered that certain mutations conferring linezolid resistance also conferred resistance to chloramphenicol. Linezolid-resistant mutants with a T to C change at position 2504 or a G to T change at position 2576 had increased MICs for both linezolid and chloramphenicol. Those mutants with T2504C and G2576T mutations displayed 4-fold and 2- to 8-fold increases in chloramphenicol MICs, respectively.

The T2504C mutation has previously been described in laboratory-selected mutants of *H. halobium*, where it conferred resistance to linezolid (Kloss *et al.,* 1999). It has also been reported to confer resistance to chloramphenicol in human and mouse mitochondrial rRNA (Blanc *et al.,* 1981a, Blanc *et al.,* 1981b). Moreover, T2504 is thought to be important for binding site of chloramphenicol in bacteria (Figure 62) (http://www.molgen.mpg.de/~agribo/agfranceschi/franceschi-projects-50S-antibiotics-cam3D.html).

If passaging in the presence of increasing concentrations of linezolid could select chloramphenicol resistance it was hypothesised that passaging strains on increasing concentrations of chloramphenicol might produce mutants resistant to both chloramphenicol and linezolid and select for the T2504C or G2576T mutations. Indeed, the resulting mutants showed a positive correlation between linezolid and chloramphenicol MICs and three mutants had G2505A and T2500A mutations. The former has previously been described in laboratory-selected linezolid-resistant enterococci (Lobritz *et al.,* 2003, Prystowsky *et al.,* 2001) and the latter has been found in clinical

Figure 62. Interaction of chloramphenicol with the ribosome of *Deinococcus radiodurans*. Diagram shows the chloramphenicol binding site at the peptidyl transferase cavity. Green represents chloramphenicol and nucleotides that interact with the antibiotic are shown with their chemical structure. Nucleotide numbering is according to the *E. coli* sequence. Putative Mg ions (Mg-C1, Mg-C2) are indicated. From http://www.molgen.mpg.de/~ag_ribo/ag_franceschi /franceschi-projects-50S-antibiotics-cam3D.html.



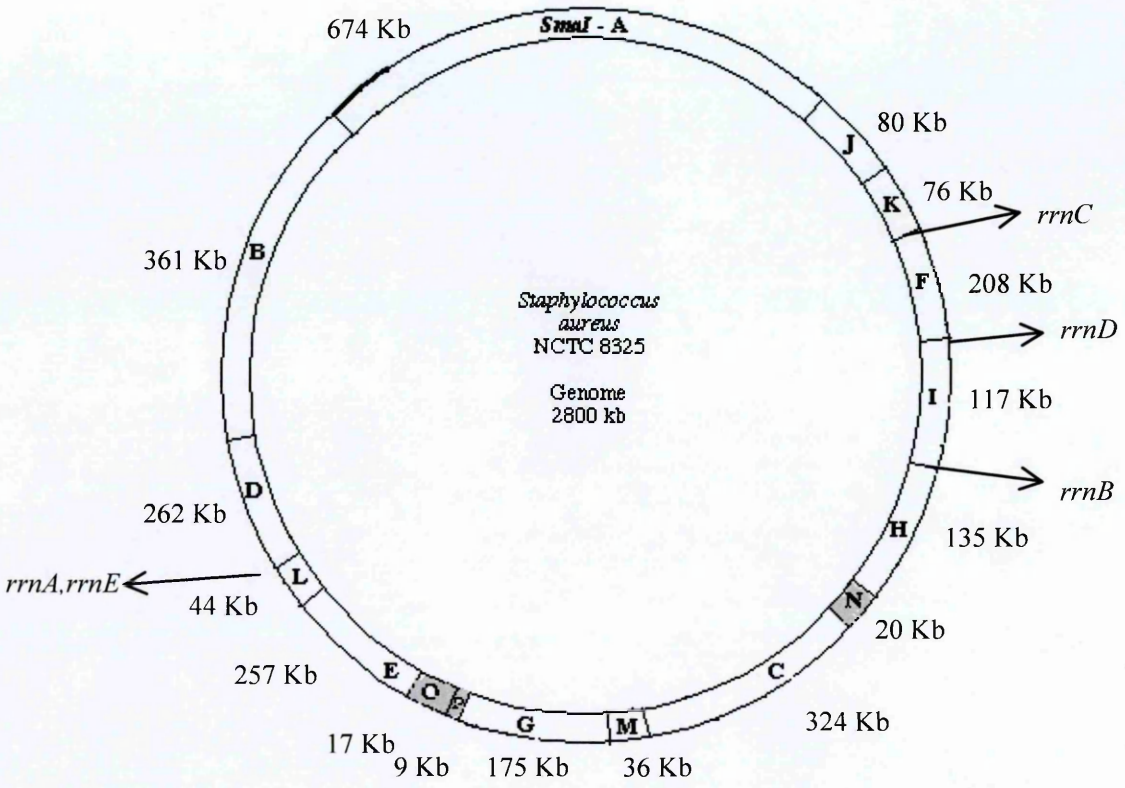
and laboratory linezolid-resistant isolates of *S. aureus* and *H. halobium* (Kloss *et al.*, 1999, Meka *et al.*, 2004b, Miller *et al.*, 2005). Surprisingly, no mutants with a T2504C mutation were detected following chloramphenicol selection. Furthermore no mutants with the G2576T mutation, most commonly found in the clinic, were detected.

When these chloramphenicol- and linezolid-resistant mutants were analysed by pyrosequencing and hybridisation it was noted that they only had one 23S rRNA gene copy mutated. These results were at odds with the chloramphenicol and linezolid MICs of the mutants (chloramphenicol MICs 8->256 mg/L). Upon determining the linezolid MICs for a second time it was discovered that the mutants were unstable and reversion towards susceptibility had occurred, even in high-level resistant mutants, before the mutants underwent pyrosequencing. These subsequent MICs (chloramphenicol and linezolid MICs 1-16 mg/L) were in agreement with the pyrosequencing results. No changes in 23S rRNA gene copy number could be detected in these three mutants.

No mutations could be detected in the remainder of the mutants where chloramphenicol resistance did not emerge or reversion to susceptibility occurred. However, if analysis by pyrosequencing had been performed before reversion to susceptibility had occurred other mutations might have been detected in the remainder of the mutants. In these mutants exposure to chloramphenicol resulted in changes in 23S rRNA gene copy number in nine of 20 mutants, with eight of the nine increasing in gene copy number by one. This increase in gene copy number echoes the results found with clinical and laboratory-selected linezolid-resistant isolates. Slight differences were apparent between the PFGE profiles of parents and chloramphenicol-resistant mutants. In one chloramphenicol- and linezolid-resistant mutant one of the *Sma*I cutting sites should have been located at the junction between *rrnA* and *rrnE* operons, however a change in the *Sma*I recognition sequence resulted in the loss of this cutting site (Figure 63). It appears that fragment D was subsequently incorporated into one of the adjacent fragments on either side of it. Exposure to chloramphenicol possibly resulted in the selection of a detectable mutation in the enzyme recognition sequence.

Cross-resistance between chloramphenicol and linezolid due to mutations in the 23S rRNA genes has not previously been reported in clinical isolates or laboratory-selected mutants of *S. aureus*. Nevertheless, laboratory studies have reported competition between the two antibiotics for

Figure 63. Restriction digest map of *S. aureus* NCTC 8325. The *rrnA* and *rrnE* operons are located between fragments D and L (Adapted from http://microgen.ouhsc.edu/s_ aureus /s _aureus _home.htm).



ribosomal binding sites in *E. coli* and *H. halobium* resulting in partial cross-resistance (Bobkova *et al.*, 2003, Kloss *et al.*, 1999, Lin *et al.*, 1997). Also, mutational resistance to chloramphenicol has previously been described in laboratory-selected isolates of *H. halobium* (Mankin and Garrett, 1991). This discovery has few clinical implications as, firstly, chloramphenicol is not widely used and secondly, resistance to chloramphenicol is usually via plasmid mediated acetylation of the drug (via the *cat* gene), which confers no cross-resistance with linezolid (Fines and Leclercq, 2000). In clinical linezolid-resistant isolates high-level chloramphenicol resistance, conferred by the *cat* gene, would mask any mutational cross-resistance between the two antibiotics.

4.1.8 Loss of erythromycin resistance with the emergence of linezolid resistance

Whilst raising linezolid-resistant mutants *in vitro*, loss of erythromycin resistance occurred in five of seven mutants of RN4220Δ*mutS*. This phenomenon has previously been seen with linezolid-resistant *S. aureus* isolates from the clinic, but is not essential for the emergence of resistance to occur (Howe *et al.*, 2002, Meka *et al.*, 2004b, Wilson *et al.*, 2003). Sakoulas *et al.*, (2003) proved that linezolid resistance can occur in combination with erythromycin resistance by successfully re-introducing an *erm* gene into a linezolid-resistant strain of *S. aureus*.

An experiment was designed to determine the frequency at which loss of erythromycin resistance would occur with the emergence of linezolid resistance, whether growing strains in the presence of linezolid and erythromycin would prevent the loss of erythromycin determinants, and whether preventing the loss of erythromycin would slow the emergence of linezolid resistance.

Results revealed that nearly half (six of 14) of all strains tested reverted to erythromycin susceptibility with the emergence of linezolid resistance. Curiously, only 25% (one of four) of mutants with an *erm*(B) determinant reverted to erythromycin susceptibility, whereas strains with an *erm*(C) determinant showed a greater propensity towards loss of an *erm* determinant in the presence of linezolid, with this occurring in 50% (five of ten) of mutants. One of the mutants that lost an *erm*(B) determinant had a G2447T mutation. However, mutations could not be detected in the 23S rRNA genes of the remaining strains in which loss of erythromycin determinants occurred. In these strains stable linezolid- resistance was not achieved (linezolid MICs of 4 mg/L), despite

repeated passage in the presence of 10 mg/L of linezolid. From these results it is apparent that on the occasions when loss of an *erm* determinant does occur, exposure to linezolid is enough to trigger the loss, full resistance to linezolid does not have to occur before loss of erythromycin occurs. Furthermore, after the loss of erythromycin resistance, linezolid resistance is not guaranteed to emerge any quicker or become any more stable. It has not yet been elucidated what advantage (if any) loss of erythromycin resistance confers on bacteria exposed to linezolid.

This work supports that of Howe *et al.*, (2002) which showed loss of erythromycin determinants can occur with the emergence of mutations other than G2576T. From previous reports concerning the loss of erythromycin determinants with the emergence of linezolid resistance in the clinic in isolates of *S. aureus* it is not apparent whether there is a propensity for one type of determinant to be lost more readily than another (Howe *et al.*, 2002, Wilson *et al.*, 2003).

It was also noted that growing erythromycin-resistant strains in the presence of linezolid and erythromycin delayed the emergence of linezolid resistance in two out of three strains, though not in the hypermutable strain (RN4220Δ*mutS*). These results imply that treating patients with erythromycin and linezolid, even if the isolate is erythromycin-resistant, might delay the emergence of linezolid resistance.

4.2 Hypermutability

In *S. aureus* resistance to linezolid and the glycopeptide antibiotics, vancomycin and teicoplanin, occurs via chromosomal mutational events. In the clinic resistance to these antibiotics is rare, but nevertheless occurs. In the laboratory resistance to these antibiotics is difficult to generate. It is plausible that, in the clinic, a hypermutable phenotype might enable the rapid generation of the chromosomal mutations required for resistance to linezolid or the glycopeptide antibiotics. Subsequent exposure to these antibiotics would co-select for resistance and hypermutability in combination.

The mutation frequencies of linezolid-resistant staphylococci selected either *in vitro* or in the clinic have not previously been determined. Therefore this study aimed to investigate whether linezolid-resistant *S. aureus* isolates from the clinic were hypermutable. The selection of linezolid-

resistant isolates in the laboratory allowed investigation into whether hypermutability was co-selected with linezolid resistance. Teicoplanin-resistant isolates from the clinic were investigated for any evidence of hypermutability. It is possible that these isolates might have a hypermutable phenotype.

4.2.1 Mutation frequencies of laboratory-selected linezolid-resistant mutants

In order to determine, firstly, whether linezolid resistance would emerge preferentially in hypermutable *S. aureus* and, secondly, whether hypermutability would be co-selected with the emergence of linezolid resistance, linezolid-resistant mutants were selected in the laboratory. A hypermutable *mutS*-negative control strain and its wild-type parent strain were used as controls to determine whether the strains tested here had mutation frequencies comparable to the hypermutable control strain and hence were themselves hypermutable.

Linezolid resistance was easier to generate in the hypermutable control strain, RN4220Δ*mutS* than the wild-type control strain, RN4220, or any of the clinical strains tested. Furthermore, the G2576T mutation was preferred in the hypermutable control strain, occurring in eight of 14 mutants. Initial mutation frequencies of the control strains RN4220Δ*mutS*, RN4220, ST/03/2121 and ST/03/2122 proved that inactivation of the *mutS* gene in *S. aureus* resulted in a hypermutable phenotype, echoing the results of O'Neill *et al.*, (2002). The linezolid resistant mutants raised in the laboratory were tested for the co-selection of linezolid resistance and hypermutability by determining the frequency with which they mutated to rifampicin and fusidic acid. The results of these mutation frequencies provided some evidence of hypermutability. A few (four of 17) of the mutants of RN4220, RN4220Δ*mutS* and ST/03/2121 had mutation frequencies to fusidic acid and rifampicin elevated above those of their parent strains, although this was more apparent for fusidic acid than for rifampicin.

The laboratory-selected linezolid-resistant mutants were further tested for any evidence of hypermutability using the fosfomycin disc test developed by Galan *et al.*, (2003, 2004). The results of the disc tests were not in agreement with the results of the mutation frequencies to rifampicin and fusidic acid, showing an overall decrease in mutability compared with parent strains. The

linezolid-resistant mutants in this study showed a decrease in the number of colonies in the zone of inhibition along with increased zone diameters when compared to their parent strains. One possible explanation for this could be decreased growth rate as a result of exposure to fosfomycin (Nilsson *et al.,* 2003). However, if this were the case then similar zone sizes and numbers of colonies in the zone of inhibition would be expected from the parent strains, instead, they were seemingly unaffected. Another explanation could be that in selecting for linezolid resistance a decreased growth rate has also been selected for.

If hypermutability had been co-selected with linezolid resistance an increase in mutation frequency to both rifampicin and fusidic acid as well as fosfomycin would be expected. The mutants tested here do not show consistent, elevated mutation frequencies to more than one antibiotic as would be expected with the co-selection of linezolid resistance and hypermutability.

4.2.2 Mutation frequencies of linezolid-resistant clinical isolates

The mutation frequencies of linezolid-resistant clinical isolates were determined to investigate whether there was any evidence for hypermutability, which could have been involved in the emergence of resistance. Evidence was lacking for the majority of strains. However, there were three isolates (3a, b and c), all of the same strain (EMRSA-15) and from the same patient, that displayed elevated mutation frequencies to rifampicin, fusidic acid and increased mutability to fosfomycin than wild-type strains and other linezolid-resistant clinical isolates (Table 31, Figure 45, Figure 46, Table 32, Figure 47 and Figure 48). This indicates hypermutability could have played a role in the emergence of linezolid resistance in this strain. This strain was not isolated from a cystic fibrosis sufferer but from the penis, perineum and a drain site of a patient.

Interestingly, six of the nine linezolid-resistant isolates from the clinic were isolated from the sputum of cystic fibrosis patients (Gales *et al.,* 2006, Hill *et al.,* 2005, Machado *et al.,* 2003). The lung of a cystic fibrosis patient is associated with the rapid development of multi-resistant strains of *S. aureus*, *P. aeruginosa* and *H. influenzae*, possibly due to hypermutability. The limited research on the role of hypermutability and the emergence of antibiotic resistance in *S. aureus* in patients with cystic fibrosis are conflicting. Prunier *et al.*, (2003) discovered that ribosomal mutations

conferring macrolide resistance in strains of *S. aureus* from cystic fibrosis patients were the most frequent mechanism of resistance, possibly due to the high proportion of hypermutable strains present (13 of 89 strains). However O'Neill *et al.*, (2002) assessed 493 *S. aureus* isolates from the clinical and community setting, including 49 strains from cystic fibrosis patients. No evidence for hypermutability was detected and it was concluded that *S. aureus* hypermutators have an extremely low clinical prevalence.

If hypermutable strains do exist in the cystic fibrosis lung and play a role in the rapid emergence of antibiotic resistance it could explain the high proportion of linezolid-resistant isolates originating from cystic fibrosis patients. However this may not be the only reason. A simple explanation is that a higher proportion of patients with cystic fibrosis are treated with linezolid and therefore resistance is more likely to occur in these isolates. Alternatively, resistance could be attributed to the variability of linezolid levels in the CF lung, with sub-therapeutic levels facilitating selection of resistance (Bosso *et al.,* 2004, Hill *et al.,* 2005, Saralaya *et al.,* 2004). Additionally, most of the published cases where linezolid resistance has occurred involve intermittent dosing, dosing at sub-therapeutic levels and long-term therapy (Meka *et al.,* 2004b, Tsiodras *et al.,* 2001, Wilson *et al.,* 2003). Perhaps it is these dosing regimens which provide the ideal opportunity for resistance to emerge and not the presence of hypermutable strains. A point to note is that inter-patient spread of *S. aureus* strains is common between cystic fibrosis sufferers and the emergence of linezolid resistance in one patient may provide the ideal opportunity for the dissemination of a linezolid-resistant strain (Gales *et al.,* 2006).

Due to the rarity of linezolid-resistant clinical isolates, this study was only able to investigate six linezolid-resistant isolates from cystic fibrosis patients. A larger group of isolates would give a more definite picture of the role, if any, of hypermutability in the emergence of linezolid resistance. In this study the linezolid-resistant isolates from cystic fibrosis patients investigated showed no evidence of hypermutability. However, the possibility of these isolates previously going through a period of transient hypermutability that cannot subsequently be detected should not be ruled out. Although stable hypermutability seems not to have played a role in the emergence of linezolid resistance the fact that such a high proportion of the resistant strains tested here are from cystic

fibrosis patients indicates that there is a link between cystic fibrosis and the emergence of linezolid resistance.

4.2.3 Mutation frequencies of teicoplanin-resistant clinical isolates

Very little work has been carried out on determining the mutation frequencies of teicoplanin-resistant clinical isolates. More work has been carried out in relation to vancomycin-resistant isolates. There is conflicting evidence as to whether these glycopeptide-resistant isolates are hypermutable. Initially it was thought that the vancomycin-intermediate resistant strain, Mu50, had an inactive *mutS* gene which was thought to have aided in the development of vancomycin resistance (Avison *et al.,* 2002) but this was later disproved (O'Neill and Chopra, 2003). Similarly, Muthaiyan *et al.*, (2004) found no mutations in the *mutS* genes of nine vancomycin-intermediate *S. aureus* clinical and laboratory strains that would result in a non-functional gene. However, Schaaff *et al.*, (2002) found that vancomycin resistance evolved quicker in a *S. aureus mutS*-negative mutant when compared with its wild-type parent strain, indicating an elevated mutation frequency, due to a defective *mutS* gene, could be involved in the emergence of resistance. Similarly, in this study, linezolid resistance was found to emerge quicker in a *mutS*-negative strain, confirming the finding of Schaaff *et al.*, (Schaaff *et al.,* 2002).

Selecting spontaneous mutants resistant to vancomycin and teicoplanin proved difficult, furthermore mutants that were selected were difficult to differentiate from each other due to clumping of colonies. However, the results obtained here were in agreement with Schaaff *et al.*, (2002) with the *mutS*-negative strain producing the highest mutation frequencies to the glycopeptide antibiotics.

A group of teicoplanin-resistant isolates from the clinic were assessed to determine whether they had a hypermutable phenotype that could have been associated with the emergence of teicoplanin resistance. Only one clinical teicoplanin-resistant isolate generated mutation frequencies to rifampicin and fusidic acid greater than those of the wild-type control, RN4220. Furthermore, it was comparable to the hypermutable control strain, RN4220Δ*mutS*, in mutability to fosfomycin. However, this group of isolates had, on average, mutation frequencies lower than linezolid-resistant

isolates from the clinic. This group of teicoplanin-resistant isolates showed no evidence of pre-existing hypermutability.

Teicoplanin-selection generated both orange and colourless mutants of the wild-type control strain, RN4220, and the hypermutable control strain, RN4220Δ*mutS*. NCTC 8325 and its descendants, RN4220 and RN4220Δ*mutS*, are natural non-pigmented mutants due to an 11 bp deletion in the gene encoding the sigmaB regulator, RsbU (Kullik *et al.,* 1998). This deletion generates a premature stop codon, consequently RN4220 and RN4220Δ*mutS* are unable to activate the RsbU-initiated cascade that leads to sigmaB activity (Giachino *et al.,* 2001). One of the properties influenced by sigmaB is pigment formation and hence RN4220 and RN4220Δ*mutS* are pigmentless (Bischoff and Berger-Bachi, 2001). Pigment formation has been found to be restored in pigmentless mutants by over-expression of sigmaB (Kullik *et al.,* 1998). Bischoff *et al.*, (2001) selected spontaneous teicoplanin-selected mutants of NCTC 8325. In the presence of teicoplanin these mutants produced an intense orange colour, usually after 48 hours. Similarly, in this study, orange pigmented mutants only emerged after 48 hours. The orange pigmented mutants were found to have point mutations in the *rsbW* gene. The RsbW protein regulates sigmaB activity; without it, excessively high sigmaB activity occurs, resulting in over expression of pigmentation and the intense orange colour (Bischoff and Berger-Bachi, 2001). Bischoff *et al.*, concluded that the *sigmaB* operon is one of the preferred mutation sites associated with first step teicoplanin resistance in pigmentless *S. aureus* strains. However, it is not known *how* a mutation in the *sigmaB* operon results in increased resistance to teicoplanin. In this study and that by Bischoff *et al.*, (2001) the presence of both white and orange colonies suggests there is another pathway to teicoplanin resistance. Additionally, both studies found orange mutants occurred in the presence of vancomycin, although this phenomenon was more pronounced in the presence of teicoplanin.

4.2.4 Hypermutability, linezolid and other antibiotic classes

Whilst investigating the phenomenon of loss of erythromycin resistance with the emergence of linezolid resistance it became apparent that linezolid resistance, in the presence and absence of erythromycin, emerged quickest in RN4220Δ*mutS*, some ten days before its comparator wild-type

strain. This indicates that pre-existing hypermutability could be a factor in the emergence of linezolid resistance. Growing this hypermutable strain in the presence of erythromycin and increasing concentrations of linezolid, unlike other erythromycin-resistant strains tested here, did not slow the emergence of resistance. It is possible that if hypermutability were a factor in the emergence of resistance, using erythromycin and linezolid against erythromycin-resistant strains would not delay the emergence of resistance.

Surprisingly when the hypermutable strain was grown in the presence of increasing concentrations of chloramphenicol, low-level chloramphenicol resistance only occurred in three of five mutants selected, whereas resistance occurred in all five mutants of its wild-type counterpart, one of which had high-level resistance. Chloramphenicol resistance was more difficult to select than linezolid resistance in the hypermutable control strain, RN4220Δ*mutS*.

4.2.5 Summary of hypermutability and the emergence of linezolid resistance

It was hypothesised that hypermutable strains would be predisposed to the emergence of antibiotic resistance. Evidence to support this theory in this work was provided by linezolid resistance emerging quicker in the hypermutable control strain than in wild-type and clinical isolates and, furthermore, in one mutant two different mutations emerged. These results indicate that if hypermutable strains were present in the clinic they would be more predisposed to developing linezolid resistance.

From the results of this study evidence against the role of stable hypermutability seems to outweigh that supporting it. The involvement of hypermutability in the emergence of linezolid resistance in clinical isolates is not proven, especially in isolates from cystic fibrosis patients. It has been proposed that strains residing in the lungs of cystic fibrosis are hypermutable and therefore more prone to chromosomal mutations which confer antibiotic resistance. There is circumstantial evidence here, with six of nine linezolid-resistant isolates studied being isolated from the lungs of cystic fibrosis patients, that hypermutability could be a plausible explanation as to why resistance emerges so quickly, especially given the evidence supporting hypermutability in *P. aeruginosa* strains from cystic fibrosis patients. However, given the mutation frequencies of

these linezolid-resistant strains, both from the clinic and those selected in the laboratory, there is no direct and convincing evidence that points towards hypermutability being involved in the emergence of resistance, nor for it being co-selected along with linezolid resistance. Furthermore, the only linezolid-resistant isolates with elevated mutation frequencies were not isolated from a patient with cystic fibrosis. There could be a simpler explanation for the emergence of resistance in strains of *S. aureus* from the cystic fibrosis lung other than hypermutability. It could be that the number of linezolid-resistant isolates from cystic fibrosis patients could reflect its greater use in these patients. Alternatively, it has been shown that penetration of antibiotics into the cystic fibrosis lung is difficult and results in variable antibiotic levels (Bosso *et al.,* 2004, Hill *et al.,* 2005, Saralaya *et al.,* 2004). This could provide strains with the ideal conditions for the emergence and selection of antibiotic resistance, especially where only one chromosomal mutation is required in the case of linezolid.

Further evidence supporting the lack of hypermutability was provided by the mutation frequencies of teicoplanin-resistant isolates, which provided no evidence of pre-existing hypermutability. If hypermutability were involved in the emergence of linezolid resistance this group of isolates would be no more prone to undergoing chromosomal mutations, resulting in the emergence of linezolid resistance.

In conclusion the evidence provided in this study supports that of O'Neill *et al.*, (2002) who proposed that there is some evidence of hypermutability in *S. aureus* strains. This was confirmed in this study by the isolation of one linezolid-resistant strain from the clinic with elevated mutation frequencies to two antibiotics, but their prevalence in the clinic is low and they may not be an important pathway to resistance as shown in other species. Hypermutability is not a prerequisite for the emergence of linezolid resistance, nor is hypermutability co-selected with linezolid resistance. However, it cannot be ruled out that the linezolid-resistant and teicoplanin-resistant isolates tested here were previously hypermutable or underwent a transient period of hypermutability when exposed to linezolid or teicoplanin that enabled them to rapidly generate mutations conferring resistance.

5 Conclusions

5.1 Conclusions

- Mutations in the 23S rRNA genes were associated with high-level resistance to linezolid in *S. aureus* isolates from the clinic and in laboratory-selected mutants. The G2576T mutation was the only mutation detected in clinical isolates and was prevalent amongst laboratory-selected mutants. Other mutations detected in mutants from the laboratory included G2447T, T2500A, A2503G, T2504C and G2505A. Mutations in the 23S rRNA genes could not be detected in five laboratory-selected mutants. Resistance selected *in vitro* was more unstable than that which had emerged in the clinic. This might be attributed to the need for a specific 23S rRNA gene copy to be mutated in order for resistance to become stable.

- Pyrosequencing successfully identified and quantified six of seven mutation types conferring resistance to linezolid. There was a positive correlation between the percentage of mutated 23S rRNA genes and the linezolid MIC. Hybridization was successfully used to quantify 23S rRNA gene copy number. The 23S rRNA gene copy number in linezolid-resistant clinical isolates of *S. aureus* ranged from four to six and from four to nine in laboratory-selected mutants. Furthermore, 23S rRNA gene copy number was affected by exposure to linezolid in half of all laboratory-selected mutants exposed to linezolid, both susceptible and resistant; the majority increased by one gene copy. Additionally, linezolid-resistant mutants were selected with no changes in 23S rRNA copy number when compared with their parent strains, but with changes in hybridization banding profile indicating changes in the restriction enzyme recognition sequence downstream of the 23S rRNA genes. Combined, the pyrosequencing and hybridization data revealed a positive correlation between the number of mutated 23S rRNA genes and the linezolid MIC.

- Cross-resistance between linezolid and chloramphenicol occurred in mutants selected either in the presence of linezolid or chloramphenicol. Mutations G2576T, T2500A, T2504C and G2505A all conferred resistance to both linezolid and chloramphenicol. Chloramphenicol-selected mutants were very unstable and reversion to susceptibility was frequent.

- Loss of *erm*-mediated erythromycin resistance occurred in nearly half of all linezolid-selected mutants. A mutation conferring resistance to linezolid was only detected in one of the mutants where reversion to susceptibility occurred. Loss of erythromycin resistance did not ensure the emergence of linezolid resistance, nor did linezolid resistance emerge quicker in those strains that had lost erythromycin determinants. There was a tendency for *erm*(C) determinants to be lost more readily than *erm*(B) determinants. There did not appear to be a correlation between the loss of resistance and the type of mutation selected. Growing erythromycin-resistant strains in the presence of erythromycin and increasing concentrations of linezolid slowed the emergence of linezolid resistance in two of three strains, but not with the hypermutable control strain. These results imply treating with linezolid and erythromycin might delay the emergence of resistance even if the isolate is erythromycin-resistant.

- Pre-existing hypermutability increased the ability of a strain to develop linezolid resistance. However, little evidence was found to suggest hypermutability is a prerequisite in the emergence of linezolid resistance either *in vitro* or in the clinic. Likewise there was little evidence for the co-selection of hypermutability and linezolid resistance in either laboratory mutants or clinical isolates. Seven of nine linezolid-resistant isolates from the clinic were isolated from patients with cystic fibrosis and although transient hypermutability could have played a role in the emergence of linezolid resistance, so too could the dosing regimens and the variability of linezolid levels in the lung. There was little evidence for hypermutability in teicoplanin-resistant isolates from the clinic.

5.2 Prospects for future studies

The finding that linezolid resistance is often unstable *in vitro* warrants further research. It would be interesting to investigate whether the emergence of stable linezolid resistance is dependent on one key 23S rRNA gene copy being mutated, anchoring resistance. The absence of mutations in such a stabilising copy might explain unstable resistance, such as that encountered in this work. Investigation as to how easy it is to select for a mutation in this stabilising copy in the laboratory would be intriguing. Furthermore, defining whether certain copies mutated before others or whether there is a sequence order by which proliferation of a mutation occurred would shed more light on linezolid resistance.

The finding that 23S rRNA gene copy number commonly changes upon exposure to linezolid provides another avenue for continuing research. It would be interesting to determine which gene copies are increased in the presence of linezolid. Duplication of the key gene copy could give maximum flexibility. However, if the regions flanking the 23S rRNA genes were identical, it would be problematic for detection as sequencing of the five published gene copies would lead to amplification of more than one copy, and unless a polymorphism was present, distinguishing identical copies would be impossible. If there were identical 23S rRNA gene copies present in resistant isolates sequencing only the five published copies would provide a misleading picture of the number of copies mutated. Furthermore, if the 23S rRNA genes and their flanking regions were identical, hybridization (to detect 23S rRNA gene copy number) would not be able to distinguish between identical copies, merely a darker area would be present after detection.

The finding of this study and others that it is possible to generate linezolid-resistant isolates with more than one type of mutation in the 23S rRNA genes provides another avenue for research. It would be interesting to determine whether two different mutations could reside on the same 23S rRNA gene copy or whether this would prove detrimental to the fitness of the mutant.

From the linezolid-resistant mutants selected in this work it is evident that mutations outside the 23S rRNA genes may be conferring resistance. Some linezolid-resistant strains from the clinic and from the laboratory had no mutations known to confer linezolid resistance. There is the possibility

that mutations in the ribosomal proteins may confer resistance. It would be intriguing to investigate the mechanisms of resistance in these mutants.

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List of websites

Website address	Date last accessed
http://www.bacterio.cict.fr/s/staphylococcus.html	28/08/06
http://141.150.157.80/bergeysoutline/outline/bergeys outline _5_2004.pdf	28/08/06
http://www.microbiology.med.umn.edu/microbiology/seminars/mmpc.html	28/08/06
http://www.harmony-microbe.net/microtyping.htm	28/08/06
http://www.bsacsurv.org/mrsweb/bacteraemia	28/08/06
http://www.rivm.nl/earss /Images/EARSS%20annual%20report%202004%20webversie_tcm61-25345.pdf	28/08/06
http://www.cdc.gov/ncidod/dhqp/ar_visavrsa_FAQ.html	28/08/06
http://www.cerexa.com/press/040506pr.html	28/08/06
http://iclaprim.com/	28/08/06
www.ncbi.nlm.nih.gov	28/08/06
http://micro-gen.ouhsc.edu/s_aureus/s_aureus_home.htm	28/08/06

Cont.

List of websites (cont.).

Website address	Date last accessed
www.basic.northwestern.edu/biotools/oligocalc.html	28/08/06
www.ncbi.nlm.nih.gov/BLAST	28/08/06
www.applied-maths.com	28/08/06
http://www.pyrosequencing.com/graphics/3019.pdf	28/08/06
http://micro-gen.ouhsc.edu/s_aureus/s_aureus_home.htm	28/08/06
http://www.molgen.mpg.de/~agribo/agfranceschi/franceschi-projects-50S-antibiotics-cam3D.html	28/08/06
http://microgen.ouhsc.edu/s_ aureus /s _aureus _home_b.htm	28/08/06

Appendix A

Table 40. Media used in this study and supplier

Media	Supplier
Brain heart infusion agar	Oxoid Ltd., Basingstoke, UK
Brain heart infusion broth	Oxoid Ltd., Basingstoke, UK
Columbia blood agar	Oxoid Ltd., Basingstoke, UK
IsoSensitest agar	Oxoid Ltd., Basingstoke, UK
IsoSensitest broth	Oxoid Ltd., Basingstoke, UK
Nutrient agar	Oxoid Ltd., Basingstoke, UK
Nutrient broth	Oxoid Ltd., Basingstoke, UK
Mueller-Hinton agar	Oxoid Ltd., Basingstoke, UK
Phosphate-buffered saline (0.01 M, pH 7.4)	HPA Media Department, London, UK
CHROMagar™ Staph aureus	CHROMagar Microbiology, Paris, France

Table 41. List of chemicals and materials used in this study and their suppliers.

Chemicals, reagents or materials	Supplier
λ DNA concatamer ladder	Bio-Rad, Hemel Hempstead, UK
123 bp DNA ladder	Invitrogen BV, Renfrew, UK
1Kb DNA ladder	Invitrogen BV, Renfrew, UK
Agarose (low gelling temperature)	FMC BioProducts, Rockland, USA
Agarose for gel electrophoresis	Helena Biosciences, Sunderland, UK
Ammonium acetate	Sigma-Aldrich, Poole, UK
Annealing buffer	Biotage AB, Uppsala, Sweden
Antibiotic discs	Oxoid, Basingstoke, UK
Anti-digoxigenin-alkaline phosphate-conjugate antibody	Roche Applied Science, Lewes, UK
Binding buffer (pyrosequencing)	Biotage AB, Uppsala, Sweden
Brij® 58-P	Sigma-Aldrich, Poole, UK
Bromophenol blue	BDH, VWR International, Poole, UK
Chloroform	BDH, VWR International, Poole, UK
dATP, dTTP, dCTP, dGTP	Invitrogen BV, Renfrew, UK
Denaturation solution (pyrosequencing)	Biotage AB, Uppsala, Sweden
Deoxycholic acid	Sigma-Aldrich, Poole, UK
DIG wash and block buffers	Roche Applied Science, Lewes, UK
DTCS quick start kit	Beckman Coulter, High Wycombe, UK
DIG-11-dUTP	Roche Applied Science, Lewes, UK
DIG-labelled DNA size marker II	Roche Applied Science, Lewes, UK
Disodium ethylene diaminotetra-acetic acid	Sigma-Aldrich, Poole, UK
Double-processed tissue culture water (PCR-quality water)	Sigma-Aldrich, Poole, UK
E test strips	BioStat, Stockport, UK
Ethanol	BDH, VWR International, Poole, UK
Ethidium bromide	Sigma-Aldrich, Poole, UK
Expand long template PCR system	Roche Applied Science, Lewes, UK
Ficoll 400	Sigma-Aldrich, Poole, UK
Glucose-6-phosphate	Sigma-Aldrich, Poole, UK
Guanidium thiocyanate	Sigma-Aldrich, Poole, UK
Hybond N blotting membrane	Amersham Biosciences, Little Chalfont, UK

Cont.

Table 41. List of chemicals and materials used in this study and their suppliers (cont.).

Chemicals, reagents or materials	Supplier
Hydrochloric acid	BDH, VWR International, Poole, UK
Lysostaphin	Sigma-Aldrich, Poole, UK
Lysozyme	Sigma-Aldrich, Poole, UK
Magnesium chloride	Sigma-Aldrich, Poole, UK
Methanol	BDH, VWR International, Poole, UK
Microfuge tubes	Eppendorf, Anachem, Luton, UK
Mineral oil	Sigma-Aldrich, Poole, UK
Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP)	Roche Applied Science, Lewes, UK
N-laurylsarcosine (Sarkosyl NL30)	BDH, VWR International, Poole, UK
PCR and sequencing primers	Sigma-Genosys, Haverhill, UK
PCR plates	Abgene, Epsom, UK
Phenol:chloroform:isoamyl alcohol	Sigma-Aldrich Aldrich, Poole, UK
Proteinase K	Sigma-Aldrich, Poole, UK
Pulsed–field certified agarose	Bio-Rad, Hemel Hempstead, UK
Pyro gold reagents	Biotage AB, Uppsala, Sweden
Pyrosequencing plates	Biotage AB, Uppsala, Sweden
Q-BIOgene geneclean turbo for PCR kit	Q-BIOgene, Cambridge, UK
Reagent cartridge (pyrosequencing)	Biotage AB, Uppsala, Sweden
Restriction endonucleases	Roche Applied Science, Lewes, UK Fermentas, Helena Biosciences, Sunderland, UK
Ribonuclease (RNase)	Sigma-Aldrich, Poole, UK
Sequencing kit (CEQTM DTCS-Quick Start)	Beckman-Coulter, High Wycombe, UK
Sodium acetate	Sigma-Aldrich, Poole, UK
Sodium chloride	Sigma-Aldrich, Poole, UK
Sodium dodecyl sulphate	Sigma-Aldrich, Poole, UK
Sodium hydroxide	Sigma-Aldrich, Poole, UK
Storage plates	Abgene, Epsom, UK
Streptavidin sepharose HP	Amersham Biosciences, Little Chalfont, UK
Taq polymerase PCR kit	Invitrogen BV, Renfrew, UK
Tris	BDH, VWR International, Poole, UK
Tri-sodium citrate	Sigma-Aldrich, Poole, UK
Washing buffer (pyrosequencing)	Biotage AB, Uppsala, Sweden

Table 42. List of apparatus used in this study and their suppliers.

Apparatus	Supplier and Manufacturers.
ADP 1200/L balance	Adam Equip. Co. Ltd., Milton Keynes, UK
Beckman CEQ8000 automated sequencer	Beckman-Coulter, High Wycombe, UK
Beckman plate centrifuge	Beckman-Coulter, High Wycombe, UK
CHEF DR®II apparatus pulsed field systems	Bio-Rad, Hemel Hempstead, UK
Cyclone vortex mixer CM1	Nickel-Electro LTD., Weston-super-Mare, UK.
DNA subcell® GT agarose gel electrophoresis system	Bio-Rad, Hemel Hempstead, UK
Dri-block™ (DB-2D)	Techme Ltd., Cambridge, UK.
Echotherm™ chilling/heating plate	Torrey Pines Scientific, San Marcos, USA
Electrophoresis constant power supply for agarose gel electrophoresis	Amersham Biosciences, Little Chalfont, UK
Electrophoresis constant power supply (EPS 500-400)	Amersham Biosciences, Little Chalfont, UK
Electrophoresis constant power supply BioRad power pack 250	Bio-Rad, Hemel Hempstead, UK
Finnpipette micropipettes	Thermo Life Sciences, Basingstoke, UK
Finnpipette multichannel pipettes	Thermo Life Sciences, Basingstoke, UK
Gilson Pipetteman	Anachem, Luton, UK.
Grant SBB6 steamer	Grant Instruments, Cambridge, UK
H4 gel electrophoresis tank	Bethesda Research Laboratories, Paisley, UK
Hybridisation oven 'Shake and Stack'	Thermo Life Sciences, Basingstoke, UK
IEC micromax centrifuge	Thermo Life Sciences, Basingstoke, UK
Mettler AE 240 balance	Mettler-Toledo, Beaumont Leys, UK
Mini-sub™ DNA electrophoresis cell	Bio-Rad, Hemel Hempstead, UK
Multipoint inoculator	Mast Diagnostics, Merseyside, UK
P x 2 thermocycler	Thermo Electron Corporation, Cambridge, UK
Pharmacia LKB vacugene XL	Amersham Biosciences, Little Chalfont, UK
pH meter (Corning model 240)	Corning Science Products, Corning, USA

Cont.

Table 42. List of apparatus used in this study and their suppliers (cont.).

Apparatus	Supplier and Manufacturers.
PSQ™ 96MA pyrosequencer	Biotage AB, Uppsala, Sweden.
Rainin pipette lite LTS multichannel	Anachem, Luton, UK.
Spectrophotometer (Densimat)	Biomerieux, Basingstoke, UK
Thermomixer comfort MTP	Eppendorf, Cambridge, UK
UV stratalinker 2400	Stratagene, California, USA.
Vacuum prep tool and workstation	Biotage AB, Uppsala, Sweden.

Table 43. Antibiotics used and their suppliers.

Antibiotic	Supplier
Chloramphenicol	Sigma
Ciprofloxacin	Bayer
Clindamycin	Sigma
Erythromycin	Sigma
Fusidic acid	Sigma
Linezolid	Pfizer
Oxacillin	Sigma
Quinupristin/dalfopristin	Aventis
Rifampicin	Sigma
Teicoplanin	Aventis
Vancomycin	Aventis
Fosfomycin	Sigma

Appendix B

Table 44. Mutation frequencies to rifampicin (50 mg/L). (See Table 13 for a summary of results)

Strain	Colony count (cfu/ml)	Av. no. of mutants in 200 µl [A]	Av. no. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
RN4220	1.08×10^{8}	1.00	5.00	4.63×10^{-8}	
	3.50×10^{8}	5.00	25.00	7.14×10^{-8}	
	2.25×10^{8}	0.50	2.50	1.11×10^{-8}	
	3.75×10^{8}	0.50	2.50	6.67×10^{-9}	
	9.50×10^{6}	0.25	1.25	1.32×10^{-7}	
	1.50×10^{8}	1.00	5.00	3.33×10^{-8}	
	1.45×10^{7}	0.50	2.50	1.72×10^{-7}	
	5.00×10^{8}	4.00	20.00	4.00×10^{-8}	
	1.75×10^{9}	3.50	17.50	1.00×10^{-8}	
	2.50×10^{8}	1.50	7.50	3.00×10^{-8}	
	2.50×10^{9}	2.50	12.50	5.00×10^{-9}	$5.07 \pm 1.65 \times 10^{-8}$
RN4220Δ*mutS*	1.03×10^{8}	19.75	98.75	9.59 x 10-7	
	4.75×10^{8}	27.00	135.00	2.84×10^{-7}	
	1.25×10^{8}	0.75	3.75	3.00×10^{-8}	
	3.00×10^{8}	46.00	230.00	7.67×10^{-7}	
	1.00×10^{8}	13.75	68.75	6.88×10^{-7}	

Cont.

Table 44. Mutation frequencies to rifampicin (50 mg/L) (cont.).

Strain	Colony count (cfu/ml)	Av. no. of mutants in 200 µl	Av. no. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
	7.50×10^{7}	13.50	67.50	9.0×10^{-7}	
	3.00×10^{8}	52.25	261.25	8.71×10^{-7}	
	1.25×10^{9}	43.75	218.75	1.82×10^{-7}	
	2.50×10^{9}	65.50	327.50	1.31×10^{-7}	$5.35 \pm 1.24 \times 10^{-7}$
ST/03/2121	6.5×10^{8}	4.00	20.00	3.08×10^{-8}	
	3.75×10^{8}	7.50	37.50	1.00×10^{-7}	
	4.75×10^{8}	2.75	13.75	2.89×10^{-8}	
	5.00×10^{8}	3.75	18.75	3.75×10^{-8}	
	1.00×10^{9}	5.00	25.00	2.50×10^{-8}	
	2.5×10^{8}	6.50	32.50	1.30×10^{-7}	$5.87 \pm 1.83 \times 10^{-8}$

[A] Average is taken from 4 x 200 µl aliquots.

Table 45. Mutation frequencies to rifampicin (50 mg/L) with sub-inhibitory concentrations of antibiotics added to broths. (See Table 13 for a summary of results)

Strain	Grown in broth containing	Colony count (cfu/ml)	Av. no. mutants in 200 µl	Av. no. mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
RN4220	0.25 mg/L cip	5.00×10^{8}	0.25	1.25	2.50×10^{-9}	
	0.25 mg/L cip	3.00×10^{6}	1.50	7.50	2.50×10^{-6}	
	0.25 mg/L cip	5.75×10^{6}	0.25	1.25	$<2.17 \times 10^{-7}$	$1.25 \pm 1.25 \times 10^{-6}$
	0.25 mg/L cip, 0.5 mg/L teic	8.50×10^{7}	0.50	2.50	2.94×10^{-8}	
	0.25 mg/L cip, 0.5 mg/L teic	5.25×10^{7}	1.00	5.00	9.52×10^{-8}	
	0.25 mg/L cip, 0.5 mg/L teic	1.60×10^{7}	0.25	1.25	$<7.81 \times 10^{-8}$	$6.23 \pm 3.29 \times 10^{-8}$
	0.25 mg/L cip, 0.5 mg/L vanc	8.00×10^{7}	0.75	3.75	4.69×10^{-8}	
	0.25 mg/L cip, 0.5 mg/L vanc	1.00×10^{7}	0.25	1.25	$<1.25 \times 10^{-7}$	
	0.25 mg/L cip, 0.5 mg/L vanc	1.25×10^{7}	2.50	12.50	1.00×10^{-6}	$5.23 \pm 4.77 \times 10^{-7}$
	0.25 mg/L cip, 0.5 mg/L lin	8.25×10^{7}	1.25	6.25	7.58×10^{-8}	
	0.25 mg/L cip, 0.5 mg/L lin	2.50×10^{7}	0.75	3.75	1.50×10^{-7}	
	0.25 mg/L cip, 0.5 mg/L lin	4.75×10^{7}	0.25	1.25	2.63×10^{-8}	$8.40 \pm 3.59 \times 10^{-8}$
RN4220Δ*mutS*	0.25 mg/L cip in broth	6.25×10^{6}	2.00	10.00	1.60×10^{-6}	
	0.25 mg/L cip in broth	1.25×10^{8}	0.75	3.75	3.00×10^{-8}	
	0.25 mg/L cip in broth	2.25×10^{7}	2.25	11.25	4.50×10^{-7}	$8.50 \pm 3.75 \times 10^{-7}$

Cont.

Table 45. Mutation frequencies to rifampicin (50 mg/L) with sub-inhibitory concentrations of antibiotics added to broths (cont.).

Strain	Grown in broth containing	Colony count (cfu/ml)	Av. no. mutants in 200 µl	Av. no. mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
	0.25 mg/L cip, 0.5 mg/L teic	1.15×10^{7}	2.25	11.25	9.78×10^{-7}	
	0.25 mg/L cip, 0.5 mg/L teic	7.50×10^{6}	0.75	3.75	9.50×10^{-7}	
	0.25 mg/L cip, 0.5 mg/L teic	3.25×10^{8}	2.00	10.00	3.08×10^{-7}	$7.45 \pm 2.19 \times 10^{-7}$
	0.25 mg/L cip, 0.5 mg/L vanc	8.75×10^{6}	2.25	11.25	1.29×10^{-6}	
	0.25 mg/L cip, 0.5 mg/L vanc	2.50×10^{7}	4.75	23.75	9.50×10^{-7}	
	0.25 mg/L cip, 0.5 mg/L vanc	1.00×10^{8}	7.00	35.00	2.33×10^{-6}	$1.54 \pm 0.41 \times 10^{-6}$
	0.25 mg/L cip, 0.5 mg/L lin	4.50×10^{7}	6.25	31.25	6.94×10^{-7}	
	0.25 mg/L cip, 0.5 mg/L lin	7.50×10^{6}	1.50	7.50	1.00×10^{-6}	
	0.25 mg/L cip, 0.5 mg/L lin	5.75×10^{7}	3.25	16.25	2.83×10^{-7}	$4.74 \pm 1.20 \times 10^{-7}$
ST/03/2121	0.25 mg/L cip in broth	7.00×10^{8}	7.50	37.50	5.36×10^{-8}	**N/A**
	0.25 mg/L cip, 0.5 mg/L teic	5.00×10^{8}	0.75	3.75	7.50×10^{-9}	**N/A**
	0.25 mg/L cip, 0.5 mg/L vanc	3.25×10^{8}	1.00	5.00	1.54×10^{-8}	**N/A**
	0.25 mg/L cip, 0.5 mg/L lin	1.75×10^{7}	0.50	2.50	1.43×10^{-7}	**N/A**

Table 46. Mutation frequencies to fusidic acid (0.5 mg/L). (See Table 13 for a summary of results)

Strain	Colony count (cfu/ml)	Av. no. mutants in 200 µl	Av. no. mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
RN4220	1.50×10^{8}	17.25	86.25	5.75×10^{-7}	
	5.0×10^{8}	81.25	406.25	8.13×10^{-7}	
	1.25×10^{9}	51.25	256.25	2.05×10^{-7}	
	1.75×10^{9}	20.50	102.50	5.86×10^{-8}	
	2.50×10^{9}	4.50	22.50	9.00×10^{-8}	$3.48 \pm 1.48 \times 10^{-7}$
RN4220Δ*mutS*	7.25×10^{8}	8.50	42.50	5.86×10^{-6}	
	1.25×10^{9}	7.75	38.75	3.10×10^{-6}	
	3.00×10^{8}	32.25	161.25	5.38×10^{-6}	
	1.25×10^{9}	63.25	316.25	2.64×10^{-6}	
	2.5×10^{9}	31.75	158.75	6.35×10^{-7}	$3.52 \pm 0.95 \times 10^{-6}$
ST/03/2121	1.75×10^{9}	67.50	337.50	1.93×10^{-7}	
	4.75×10^{8}	60.50	302.50	6.37×10^{-7}	
	3.75×10^{8}	54.50	272.50	7.27×10^{-7}	
	5.00×10^{8}	57.25	286.25	5.73×10^{-7}	
	1.00×10^{9}	63.00	315.00	3.15×10^{-7}	
	2.50×10^{8}	26.25	131.25	5.25×10^{-7}	$4.95 \pm 0.83 \times 10^{-7}$

Cont.

Table 46. Mutation frequencies to fusidic acid (0.5 mg/L) (cont.).

Strain	Colony count (cfu/ml)	Av. no. mutants in 200 µl	Av. no. mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
ST/03/2122	5.00×10^{8}	2.25	11.25	2.25×10^{-8}	
	2.25×10^{8}	10.25	51.25	2.28×10^{-7}	
	1.00×10^{9}	2.75	13.75	1.38×10^{-8}	
	2.50×10^{8}	3.25	16.25	6.5×10^{-8}	
	1.75×10^{9}	4.00	20.00	1.14×10^{-8}	
	7.50×10^{9}	5.75	28.75	3.83×10^{-9}	$5.74 \pm 3.52 \times 10^{-8}$

Table 47. Mutation frequencies to teicoplanin (6 mg/L). (See Table 13 for a summary of results)

Strain	Colony count (cfu/ml)	Av. no. mutants in 200 µl	Av. no. mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
RN4220	2.50×10^{8}	5.50	27.5	1.10×10^{-7}	
	3.75×10^{8}	0.25	1.25	$<3.33 \times 10^{-9}$	
	1.45×10^{7}	0.25	1.25	$<8.62 \times 10^{-8}$	
	5.00×10^{8}	4.25	21.25	4.25×10^{-8}	$7.63 \pm 3.38 \times 10^{-8}$
RN4220Δ*mutS*	7.25×10^{7}	73.5	367.5	5.07×10^{-6}	
	3.0×10^{8}	5.00	25.00	8.33×10^{-8}	
	1.45×10^{7}	2.50	12.50	8.62×10^{-7}	
	3.0×10^{8}	4.50	22.50	7.50×10^{-8}	$1.52 \pm 1.20 \times 10^{-6}$
ST/03/2121	6.50×10^{8}	7.33	36.65	5.64×10^{-8}	
	4.75×10^{8}	8.00	40.00	8.42×10^{-8}	$7.03 \pm 1.39 \times 10^{-8}$

Table 48. Mutation frequencies to vancomycin (6 mg/L). (See Table 13 for a summary of results)

Strain	Colony count (cfu/ml)	Av. no. mutants in 200 µl	Av. no. mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
RN4220	2.50×10^{8}	0.50	2.50	1.00×10^{-8}	
	1.45×10^{7}	0.25	1.25	$<8.62 \times 10^{-8}$	
	5.00×10^{8}	0.25	1.25	2.5×10^{-9}	
	3.75×10^{8}	0.50	2.50	6.67×10^{-9}	$6.39 \pm 2.17 \times 10^{-9}$
RN4220Δ*mutS*	7.25×10^{7}	0.75	3.75	5.17×10^{-8}	
	3.00×10^{8}	2.00	10.00	3.33×10^{-8}	
	1.10×10^{7}	14.25	71.25	6.48×10^{-6}	
	1.45×10^{7}	1.50	7.50	5.17×10^{-7}	
	3.00×10^{8}	0.25	1.25	4.17×10^{-9}	$1.42 \pm 1.27 \times 10^{-6}$
ST/03/2121	6.50×10^{8}	0.33	1.65	2.54×10^{-9}	**N/A**

Table 49. Mutation frequencies to linezolid (6 mg/L). (See Table 13 for a summary of results)

Strain	Grown in the presence of	Colony count (cfu/ml)	Av. no. of mutants in 200 µl	Av. no. of mutants in 1 ml	Mutation frequency
RN4220	N/A	1.08×10^{8}	0.25	1.25	$<1.16 \times 10^{-8}$
	N/A	2.50×10^{8}	0.25	1.25	$<5.00 \times 10^{-9}$
	N/A	3.75×10^{7}	0.25	1.25	$<3.33 \times 10^{-9}$
	0.5 mg/L lin in broth	1.03×10^{8}	0.25	1.25	$<1.21 \times 10^{-8}$
	0.5 mg/L lin in broth	7.25×10^{7}	0.25	1.25	$<1.72 \times 10^{-9}$
	0.5 mg/L lin in broth	4.75×10^{7}	0.25	1.25	$<2.63 \times 10^{-8}$
RN4220Δ*mutS*	N/A	1.03×10^{8}	0.25	1.25	$<1.21 \times 10^{-8}$
	N/A	1.03×10^{8}	0.25	1.25	$<1.14 \times 10^{-8}$
	N/A	6.50×10^{7}	0.25	1.25	$<1.92 \times 10^{-8}$
	0.5 mg/L lin in broth	1.03×10^{8}	0.25	1.25	$<1.14 \times 10^{-8}$
	0.5 mg/L lin in broth	6.50×10^{7}	0.25	1.25	$<1.92 \times 10^{-8}$
	0.5 mg/L lin in broth	5.75×10^{7}	0.25	1.25	$<2.17 \times 10^{-8}$
ST/03/2121	N/A	6.5×10^{8}	0.25	1.25	$<1.92 \times 10^{-9}$
	0.5 mg/L lin in broth	2.25×10^{8}	0.25	1.25	$<5.56 \times 10^{-9}$
ST/03/2122	N/A	3.50×10^{8}	0.25	1.25	$<3.75 \times 10^{-9}$
	0.5 mg/L lin in broth	3.75×10^{8}	0.25	1.25	$<3.33 \times 10^{-9}$

Table 50. Mutation frequencies of laboratory-selected linezolid-resistant isolates to rifampicin (50 mg/L). (See Table 21 for a summary of results)

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
RN4220	1.75×10^{9}	3.50	17.50	1.00×10^{-8}	
	2.50×10^{8}	1.50	7.50	3.00×10^{-8}	
	2.50×10^{9}	5.00	25.00	1.00×10^{-8}	$1.67 \pm 0.67 \times 10^{-8}$
M1	2.50×10^{8}	1.25	6.25	2.50×10^{-8}	
	5.00×10^{8}	2.75	13.75	2.75×10^{-8}	
	2.50×10^{8}	3.50	17.50	7.00×10^{-8}	$4.08 \pm 1.46 \times 10^{-8}$
M2	2.50×10^{8}	1.50	7.50	3.00×10^{-8}	
	1.50×10^{9}	4.25	21.25	1.42×10^{-8}	
	2.50×10^{8}	1.50	7.50	3.00×10^{-8}	$2.47 \pm 0.53 \times 10^{-8}$
M3	1.25×10^{8}	9.50	47.50	3.80×10^{-7}	
	2.50×10^{8}	1.50	7.50	3.00×10^{-8}	
	1.00×10^{9}	5.00	25.00	2.50×10^{-8}	$1.45 \pm 1.18 \times 10^{-7}$
RN4220Δ*mutS*	1.25×10^{9}	43.75	218.75	1.75×10^{-7}	
	2.50×10^{9}	65.50	327.50	1.31×10^{-7}	
	2.50×10^{9}	76.00	380.00	1.52×10^{-7}	$1.53 \pm 0.13 \times 10^{-7}$

Cont.

Table 50. Mutation frequencies of laboratory-selected linezolid-resistant isolates to rifampicin (50 mg/L) (cont.).

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
M4	1.25×10^{9}	1.25	6.25	5.00×10^{-9}	
	7.50×10^{8}	4.50	22.50	3.00×10^{-8}	
	7.50×10^{8}	1.50	7.50	1.00×10^{-8}	$1.50 \pm 0.76 \times 10^{-8}$
M5	2.50×10^{8}	2.25	11.25	4.50×10^{-8}	
	1.25×10^{9}	1.00	5.00	4.00×10^{-9}	
	1.50×10^{9}	0.50	2.50	1.67×10^{-9}	$1.69 \pm 1.41 \times 10^{-8}$
M6	2.50×10^{8}	1.25	6.25	2.50×10^{-8}	
	7.50×10^{8}	7.00	35.00	4.67×10^{-8}	
	2.50×10^{8}	9.00	45.00	1.80×10^{-7}	$8.39 \pm 4.85 \times 10^{-8}$
M7	7.50×10^{8}	14.00	70.00	9.33×10^{-8}	
	7.50×10^{8}	3.25	16.25	2.17×10^{-8}	
	1.30×10^{9}	14.00	70.00	5.38×10^{-8}	$5.63 \pm 2.07 \times 10^{-8}$
M8	1.25×10^{9}	69.00	345.00	2.76×10^{-7}	
	1.00×10^{9}	5.50	27.50	2.75×10^{-8}	
	4.50×10^{8}	11.50	57.50	1.28×10^{-7}	$1.44 \pm 0.72 \times 10^{-7}$
M9	2.50×10^{8}	29.25	146.25	5.85×10^{-7}	
	2.50×10^{9}	43.75	218.75	8.75×10^{-8}	
	1.25×10^{9}	40.25	201.25	1.61×10^{-7}	$2.78 \pm 1.55 \times 10^{-7}$

Cont.

Table 50. Mutation frequencies of laboratory-selected linezolid-resistant isolates to rifampicin (50 mg/L) (cont.).

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
M10	3.75×10^{8}	28.75	143.75	3.83×10^{-7}	
	1.75×10^{9}	30.25	151.25	8.64×10^{-8}	
	5.00×10^{8}	48.00	240.00	4.80×10^{-7}	$3.17 \pm 1.18 \times 10^{-7}$
ST/03/2121	2.50×10^{8}	3.75	18.75	7.50×10^{-8}	
	1.00×10^{9}	5.00	25.00	2.50×10^{-8}	
	2.50×10^{9}	6.50	32.50	1.30×10^{-8}	$3.77 \pm 1.90 \times 10^{-8}$
M11	5.00×10^{8}	5.75	28.75	5.75×10^{-8}	
	1.00×10^{9}	3.50	17.50	1.75×10^{-8}	
	5.00×10^{8}	4.00	20.00	4.00×10^{-8}	$3.83 \pm 1.16 \times 10^{-8}$
M12	2.50×10^{8}	3.75	18.75	7.50×10^{-8}	
	2.50×10^{8}	2.50	12.50	5.00×10^{-8}	
	5.00×10^{8}	3.50	17.50	3.50×10^{-8}	$5.33 \pm 1.17 \times 10^{-8}$
M13	5.00×10^{8}	5.25	26.25	5.25×10^{-8}	
	2.50×10^{9}	4.25	21.25	8.50×10^{-9}	
	7.50×10^{8}	2.00	10.00	1.33×10^{-8}	2.48 ±1.39 x 10-8
M14	1.25×10^{9}	10.50	52.50	4.20×10^{-8}	
	1.50×10^{9}	3.25	16.25	1.08×10^{-8}	
	2.50×10^{8}	2.50	12.50	5.00×10^{-8}	3.43 ±1.19 x 10-8

Cont.

Table 50. Mutation frequencies of laboratory-selected linezolid-resistant isolates to rifampicin (50 mg/L) (cont.).

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
M15	5.00×10^{9}	2.50	12.50	2.50×10^{-9}	
	2.50×10^{9}	6.50	32.50	1.30×10^{-8}	
	1.25×10^{9}	3.75	18.75	1.50×10^{-8}	1.02 ±0.39 x 10-8
M16	1.00×10^{9}	2.00	10.00	1.00×10^{-8}	
	2.50×10^{9}	2.25	11.25	4.50×10^{-9}	
	7.50×10^{8}	0.50	2.50	3.33×10^{-9}	5.94 ±2.06 x 10-8
M17	1.50×10^{9}	5.00	25.00	1.67×10^{-8}	
	7.50×10^{8}	8.25	41.25	5.50×10^{-8}	
	5.00×10^{8}	2.00	10.00	2.00×10^{-8}	3.06 ±1.23 x 10-8

Table 51. Mutation frequencies of laboratory-selected linezolid-resistant isolates to fusidic acid (0.5 mg/L). (See Table 21 for a summary of results)

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
RN4220	1.75×10^{9}	20.50	102.50	5.86×10^{-8}	
	2.50×10^{8}	31.00	155.00	6.20×10^{-7}	
	2.50×10^{9}	45.00	225.00	9.00×10^{-8}	$2.56 \pm 1.82 \times 10^{-7}$
M1	2.50×10^{8}	15.50	77.50	3.10×10^{-7}	
	5.00×10^{8}	34.25	171.25	3.43×10^{-7}	
	2.50×10^{8}	26.50	132.50	5.30×10^{-7}	$3.94 \pm 0.69 \times 10^{-7}$
M2	2.50×10^{8}	28.00	140.00	5.60×10^{-7}	
	1.50×10^{9}	258.00	1290.00	8.60×10^{-7}	
	2.50×10^{8}	3.00	15.00	6.00×10^{-8}	$4.93 \pm 2.33 \times 10^{-7}$
M3	1.25×10^{8}	7.75	38.75	3.10×10^{-7}	
	2.50×10^{8}	5.67	28.35	3.10×10^{-7}	
	1.00×10^{9}	11.00	55.00	5.50×10^{-8}	$1.59 \pm 0.77 \times 10^{-7}$
M4	1.25×10^{9}	22.50	112.50	9.00×10^{-8}	
	7.50×10^{8}	30.00	150.00	2.00×10^{-7}	
	7.50×10^{8}	30.00	150.00	2.00×10^{-7}	$1.63 \pm 0.37 \times 10^{-7}$

Cont.

Table 51. Mutation frequencies of laboratory-selected linezolid-resistant isolates to fusidic acid (0.5 mg/L) (cont.)

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
M5	2.50×10^{8}	12.50	62.50	2.50×10^{-7}	
	1.25×10^{9}	3.30	16.50	1.32×10^{-8}	
	1.50×10^{9}	37.50	187.50	1.25×10^{-7}	$1.29 \pm 0.68 \times 10^{-7}$
M6	2.50×10^{8}	77.50	387.50	1.55×10^{-6}	
	7.50×10^{8}	16.67	83.35	1.11×10^{-7}	
	2.50×10^{8}	27.50	137.50	5.50×10^{-7}	7.37 ±4.26 x 10-7
M7	7.50×10^{8}	42.50	212.50	2.83×10^{-7}	
	7.50×10^{8}	10.00	50.00	6.67×10^{-8}	
	1.30×10^{9}	20.00	100.00	7.69×10^{-8}	1.42 ±0.71 x 10-7
M8	1.25×10^{9}	16.00	80.00	6.40×10^{-8}	
	1.00×10^{9}	23.30	116.50	1.17×10^{-7}	
	4.50×10^{8}	45.00	225.00	5.00×10^{-7}	2.27 ±1.37 x 10-7
RN4220Δ*mutS*	1.25×10^{9}	320.00	1600.00	1.28×10^{-6}	
	2.50×10^{9}	633.00	3165.00	1.27×10^{-6}	
	2.50×10^{9}	317.50	1587.50	6.35×10^{-7}	1.06 ±0.21 x 10-6
M9	2.50×10^{8}	70.00	350.00	1.40×10^{-6}	
	2.50×10^{9}	486.00	100.00	4.00×10^{-8}	
	1.25×10^{9}	13.75	68.75	5.50×10^{-8}	4.98 ±4.51 x 10-7

Cont.

Table 51. Mutation frequencies of laboratory- selected linezolid-resistant isolates to fusidic acid (0.5 mg/L) (cont.)

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
M10	3.75×10^{8}	95.00	475.00	1.27×10^{-6}	
	1.75×10^{9}	156.70	783.50	4.48×10^{-7}	
	5.00×10^{8}	340.00	1700.00	3.40×10^{-6}	1.70 ±0.88 x 10-6
ST/03/2121	2.50×10^{8}	57.25	286.25	1.15×10^{-6}	
	1.00×10^{9}	63.00	315.00	3.15×10^{-7}	
	2.50×10^{9}	26.25	131.25	5.25×10^{-8}	5.04 ±3.29 x 10-7
M11	5.00×10^{8}	22.50	112.50	5.50×10^{-7}	
	1.00×10^{9}	17.75	88.75	8.88×10^{-8}	
	5.00×10^{8}	25.50	127.50	5.50×10^{-7}	1.90 ±0.51 x 10-7
M12	2.50×10^{8}	18.75	93.75	3.75×10^{-7}	
	2.50×10^{8}	24.25	121.25	4.85×10^{-7}	
	5.00×10^{8}	9.75	48.75	9.75×10^{-8}	3.19 ±1.15 x 10-7
M13	5.00×10^{8}	28.30	141.50	2.83×10^{-7}	
	2.50×10^{9}	46.00	230.00	9.20×10^{-8}	
	7.50×10^{8}	8.00	40.00	5.33×10^{-8}	1.43 ±0.71 x 10-7
M14	1.25×10^{9}	39.50	197.50	1.58×10^{-7}	
	1.50×10^{9}	20.50	102.50	6.83×10^{-8}	
	2.50×10^{8}	8.50	42.50	1.70×10^{-7}	1.32 ±0.32 x 10-7

Cont.

Table 51. Mutation frequencies of laboratory-selected linezolid-resistant isolates to fusidic acid (0.5 mg/L)(cont.)

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
M15	5.00×10^{9}	33.25	166.25	3.33×10^{-8}	
	2.50×10^{9}	38.00	190.00	7.60×10^{-8}	
	1.25×10^{9}	34.00	170.00	1.36×10^{-7}	8.18 ±2.98 x 10-8
M16	1.00×10^{9}	10.50	52.50	5.25×10^{-8}	
	2.50×10^{9}	145.00	725.00	2.90×10^{-7}	
	7.50×10^{8}	1.25	6.25	8.33×10^{-9}	1.17 ±0.88 x 10-7
M17	1.50×10^{9}	30.00	150.00	1.00×10^{-7}	
	7.50×10^{8}	17.50	87.50	1.17×10^{-7}	
	5.00×10^{8}	16.00	80.00	1.60×10^{-7}	1.26 ±0.18 x 10-7

Table 52. Number of days taken for a mutant to grow at each concentration of linezolid. (See Table 25 for a summary of results)

			No. of days at each linezolid concentration							
Mutant	**Parent**	**Grown in the presence of**	**1 mg/L**	**2 mg/L**	**4 mg/L**	**6 mg/L**	**8 mg/L**	**10 mg/L**	**Total time taken**	**Average time taken**
M1	RN4220	Lin	3	18	5	4	3	1	34	
M2	RN4220	Lin	2	2	2	6	5	1	18	
M3	RN4220	Lin	2	2	35	4	5	1	49	
M34	RN4220	Lin	3	7	19	6	5	1	41	35.5
M4	RN4220Δ*mutS*	Lin	3	3	15	5	4	1	31	
M5	RN4220Δ*mutS*	Lin	2	2	2	8	3	1	18	
M6	RN4220Δ*mutS*	Lin	2	2	2	14	5	1	26	
M35	RN4220Δ*mutS*	Lin	2	5	6	9	3	1	26	25.3
M7	RN4220Δ*mutS*	Lin and ery (100 mg/L)	3	3	15	5	4	1	31	
M8	RN4220Δ*mutS*	Lin and ery (100 mg/L)	2	2	2	6	5	1	18	
M9	RN4220Δ*mutS*	Lin and ery (100 mg/L)	2	2	2	6	5	1	18	
M36	RN4220Δ*mutS*	Lin and ery (8 mg/L)	2	4	7	12	4	1	30	24.3

Cont.

Table 52. Number of days taken for a mutant to grow at each concentration of linezolid (cont.).

			No. of days at each linezolid concentration							
Mutant	**Parent**	**Grown in the presence of**	**1mg/L**	**2mg/L**	**4 mg/L**	**6 mg/L**	**8 mg/L**	**10 mg/L**	**Total time taken**	**Average time taken**
M10	7499	Lin	3	11	7	24	4	1	50	
M11	7499	Lin	2	2	50	3	6	1	64	
M12	7499	Lin	2	2	51	9	5	1	70	
M13	7499	Lin	2	6	8	2	5	1	24	
M37	7499	Lin	3	8	31	6	5	1	54	52.4
M14	7500	Lin	3	3	24	3	3	1	37	
M15	7500	Lin	2	2	53	3	6	1	67	
M16	7500	Lin	2	2	2	14	5	1	26	
M17	7500	Lin	2	6	2	6	2	1	19	
M38	7500	Lin	2	7	29	8	4	1	51	40.0
M18	7500	Lin and ery (100 mg/L)	3	18	9	3	3	1	37	
M19	7500	Lin and ery (100 mg/L)	2	2	53	11	4	1	73	
M20	7500	Lin and ery (100 mg/L)	2	2	48	1	5	1	59	
M21	7500	Lin and ery (100 mg/L)	2	6	24	9	5	1	47	
M39	7500	Lin and ery (8 mg/L)	3	9	34	7	4	1	58	54.8

Cont.

Table 52. Number of days taken for a mutant to grow at each concentration of linezolid (cont.).

			No. of days at each linezolid concentration							
Mutant	**Parent**	**Grown in the presence of**	**1mg/L**	**2mg/L**	**4mg/L**	**6mg/L**	**8 mg/L**	**10 mg/L**	**Total time taken**	**Average time taken**
M22	7717	Lin	3	11	7	5	7	1	34	
M23	7717	Lin	2	2	2	6	5	1	18	
M24	7717	Lin	2	2	51	6	8	1	70	
M25	7717	Lin	2	6	2	13	2	1	26	
M40	7717	Lin	3	9	42	8	9	1	72	44.0
M26	7501	Lin	3	5	13	5	10	1	37	
M27	7501	Lin	2	2	30	8	3	1	46	
M28	7501	Lin	2	2	31	17	11	1	64	
M29	7501	Lin	2	6	2	8	3	1	22	
M41	7501	Lin	2	5	16	9	8	1	41	42.0
M30	7501	Lin and ery (100 mg/L)	3	3	48	5	3	1	63	
M31	7501	Lin and ery (100 mg/L)	2	2	53	15	6	1	79	
M32	7501	Lin and ery (100 mg/L)	2	2	44	3	7	1	59	
M33	7501	Lin and ery (100 mg/L)	2	6	8	2	7	1	26	
M42	7501	Lin and ery (8 mg/L)	3	7	36	12	6	1	65	58.4

Figure 64. Loss of erythromycin resistance with the emergence of linezolid resistance, experiment 1. (See Figure 35 for a summary of results)

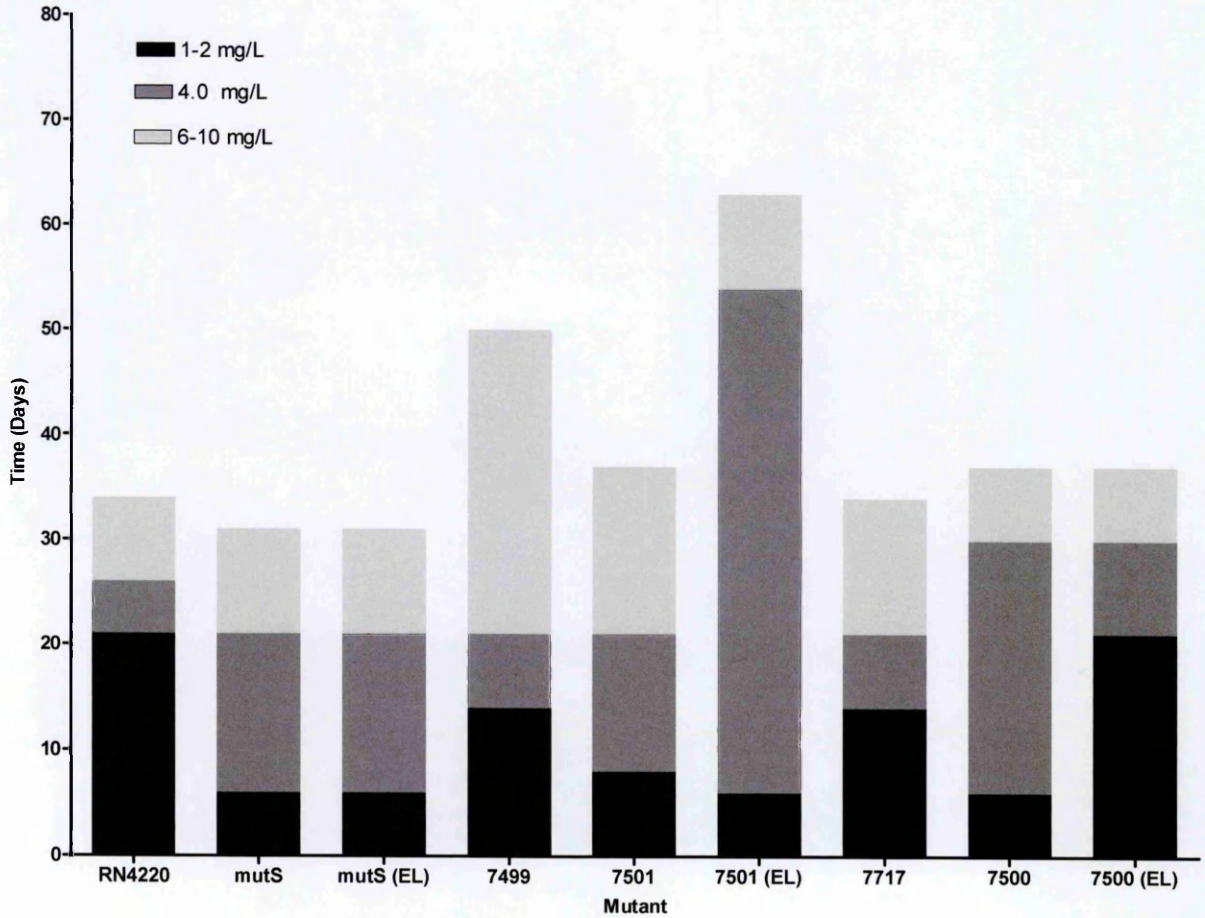

Figure 65. Loss of erythromycin resistance with the emergence of linezolid resistance, experiment 2. (See Figure 35 for a summary of results)

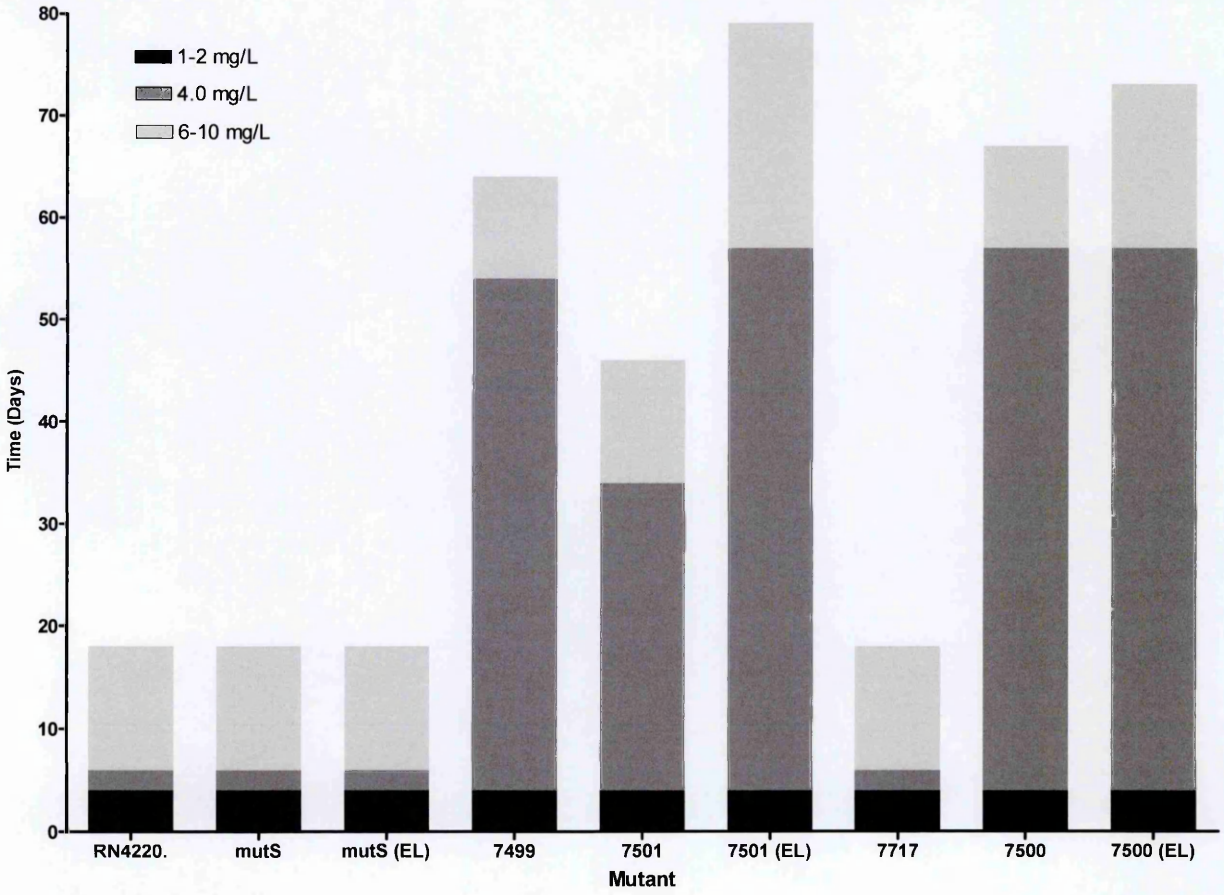

Figure 66. Loss of erythromycin resistance with the emergence of linezolid resistance, experiment 3. (See Figure 35 for a summary of results)

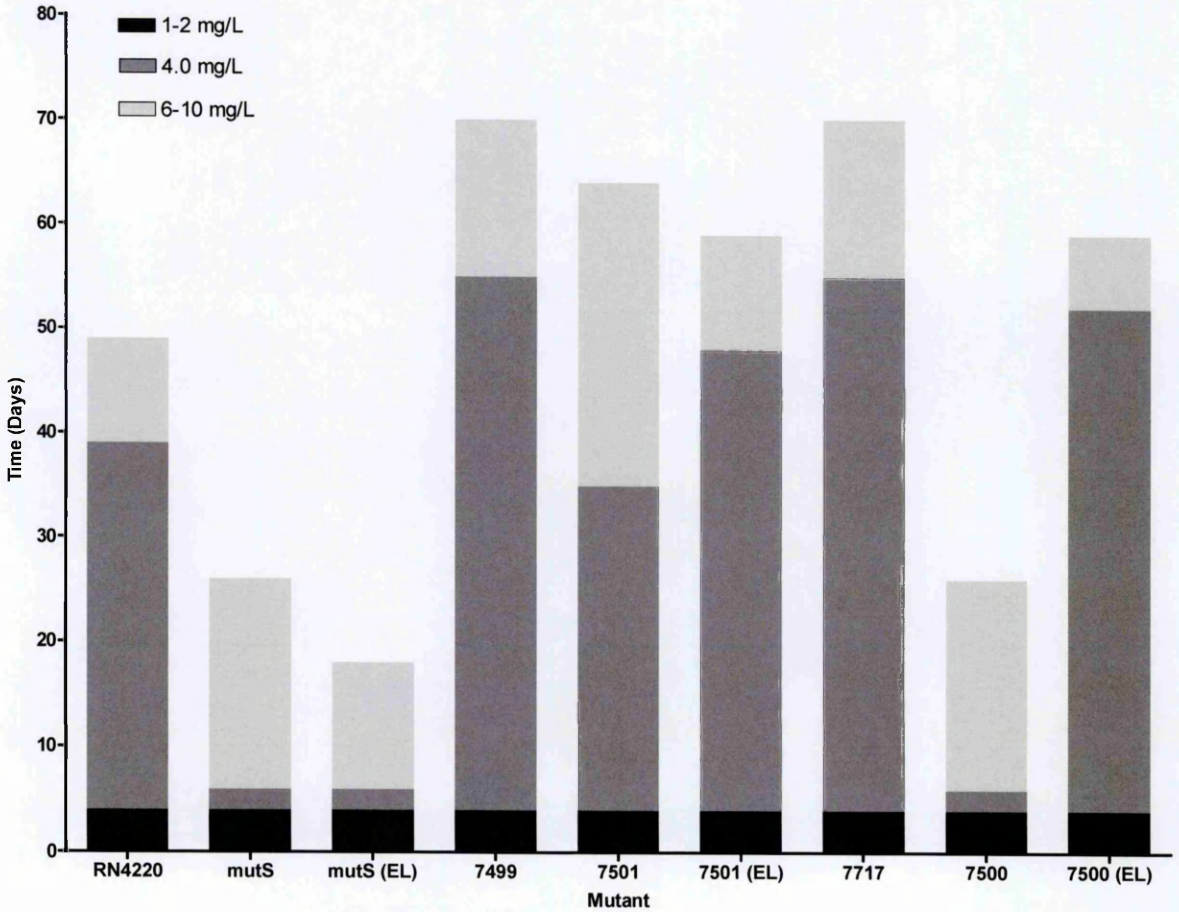

Figure 67. Loss of erythromycin resistance with the emergence of linezolid resistance, experiment 4. (See Figure 35 for a summary of results)

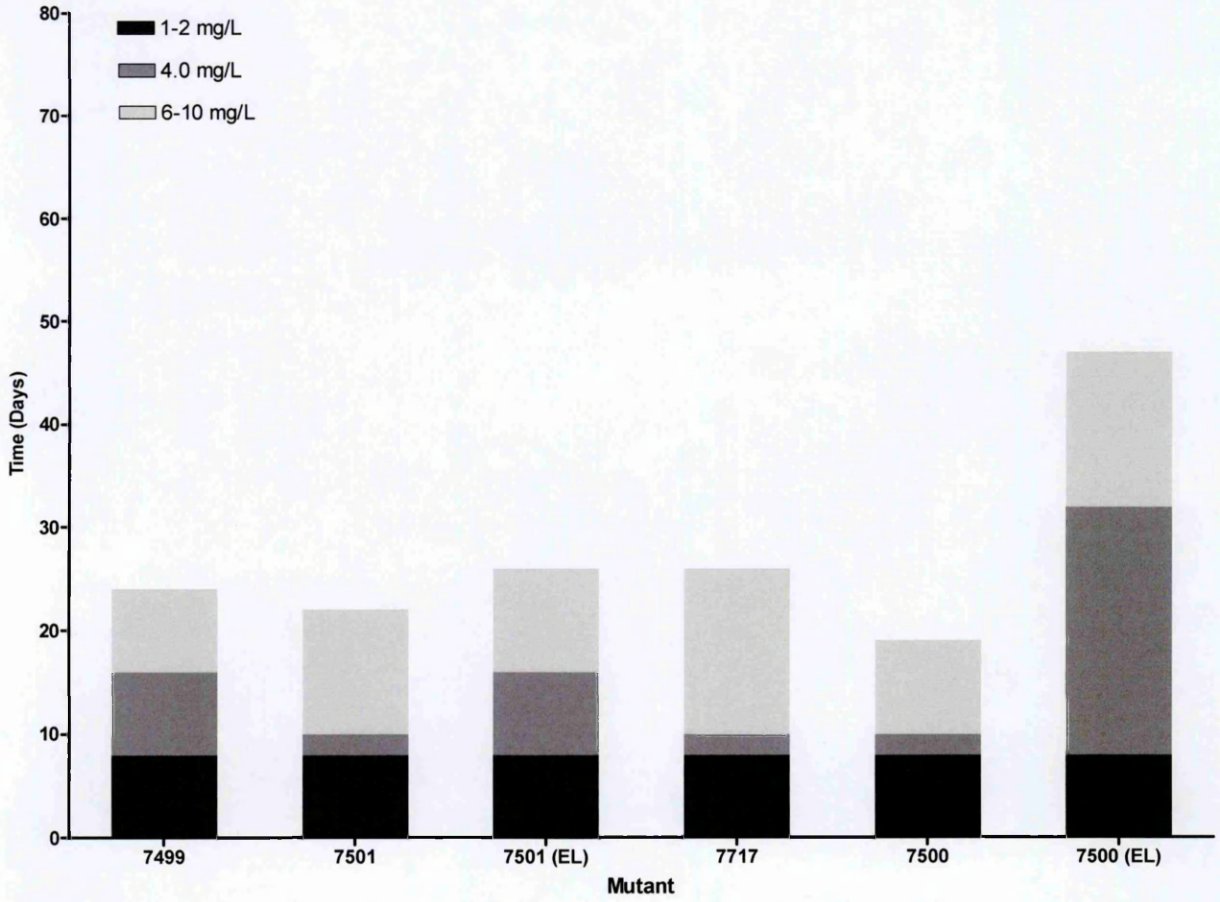

Table 53. Mutation frequencies of clinical linezolid-resistant isolates to rifampicin at 4 x MIC (see Table 31 for a summary of results)

Patient no.	Isolate	Colony count (cfu/ml)	No. of mutants in 200 µl	No. of mutants in 1 ml	Mutation frequency	Average mutation freq and SD
	RN4220	7.5×10^{9}	12.00	60.00	8×10^{-9}	$1.37 \pm 0.33 \times 10^{-8}$
		2.50×10^{9}	9.75	48.75	1.95×10^{-8}	
		2.50×10^{9}	6.75	33.75	1.35×10^{-8}	
	RN4220Δ*mutS*	1.50×10^{9}	6.00	30.00	2.00×10^{-6}	$1.18 \pm 0.41 \times 10^{-6}$
		2.5×10^{9}	4.25	21.25	8.50×10^{-7}	
		2.50×10^{9}	3.50	17.50	7.00×10^{-7}	
	ST/03/2121	5.00×10^{9}	15.00	75.00	1.50×10^{-8}	$1.99 \pm 0.34 \times 10^{-8}$
		2.50×10^{9}	13.25	66.25	2.65×10^{-8}	
		5.00×10^{9}	18.25	91.25	1.83×10^{-8}	
	MSSA	2.50×10^{9}	8.75	43.75	1.75×10^{-8}	$1.34 \pm 0.21 \times 10^{-8}$
		5.00×10^{9}	10.75	53.75	1.08×10^{-8}	
		5.00×10^{9}	12.00	60.00	1.20×10^{-8}	
	EMRSA-15	5.00×10^{9}	11.50	57.50	1.15×10^{-8}	
		2.50×10^{9}	9.50	47.50	1.90×10^{-8}	
		2.75×10^{8}	8.50	42.50	1.55×10^{-7}	$6.17 \pm 4.65 \times 10^{-8}$

Cont.

Table 53. Mutation frequencies of clinical linezolid-resistant isolates to rifampicin at 4 x MIC (cont.).

Patient no.	Isolate	Colony count (cfu/ml)	No. of mutants in 200 µl	No. of mutants in 1 ml	Mutation frequency	Average mutation freq and SD
	EMRSA-16	2.50×10^{9}	14.00	70.00	2.80×10^{-8}	
		7.50×10^{9}	10.75	53.75	7.17×10^{-9}	
		1.25×10^{10}	11.00	55.00	4.40×10^{-9}	$1.32 \pm 0.75 \times 10^{-8}$
3a	**H042800236**	1.75×10^{9}	10.00	50.00	2.86×10^{-8}	
		7.50×10^{8}	16.25	81.25	1.08×10^{-7}	
		5.00×10^{9}	19.25	96.25	1.93×10^{-8}	$5.21 \pm 2.83 \times 10^{-8}$
3b	**H042800257**	2.50×10^{9}	21.00	105.00	4.20×10^{-8}	
		2.50×10^{9}	22.00	110.00	4.40×10^{-8}	
		7.50×10^{8}	30.75	153.75	2.05×10^{-7}	$9.70 \pm 5.40 \times 10^{-8}$
3c	**H042800258**	2.50×10^{9}	19.00	95.00	3.80×10^{-8}	
		1.50×10^{9}	15.50	77.50	5.17×10^{-8}	
		2.50×10^{9}	13.25	66.25	2.65×10^{-8}	$3.87 \pm 0.73 \times 10^{-8}$
4a	**H053760467**	5.00×10^{9}	19.25	96.25	1.93×10^{-8}	
		7.50×10^{9}	14.50	72.50	9.67×10^{-9}	
		1.75×10^{9}	23.00	115.00	6.57×10^{-8}	$3.15 \pm 1.73 \times 10^{-8}$
4b	**H053760468**	5.00×10^{9}	18.25	91.25	1.83×10^{-8}	
		1.75×10^{9}	22.75	113.75	6.50×10^{-8}	
		2.50×10^{9}	18.50	92.50	3.70×10^{-8}	$4.01 \pm 1.36 \times 10^{-8}$

Table 54. Mutation frequencies of clinical linezolid-resistant isolates to fusidic acid at 4 x MIC (see Table 31 for a summary of results)

Patient no.	Isolate	Colony count (cfu/ml)	No. of mutants in 200 µl	No. of mutants in 1 ml	Mutation frequency	Average mutation freq and SD
	RN4220	7.50×10^{8}	47.50	237.50	3.17×10^{-7}	
		7.50×10^{8}	48.75	243.75	3.25×10^{-7}	
		2.50×10^{9}	53.50	267.50	1.07×10^{-7}	$2.50 \pm 0.71 \times 10^{-7}$
	RN4220Δ*mutS*	5.00×10^{8}	1400.00	7000.00	1.40×10^{-5}	
		1.50×10^{9}	825.00	4125.00	2.75×10^{-6}	
		2.50×10^{9}	1125.00	5625.00	2.25×10^{-6}	$6.33 \pm 3.84 \times 10^{-6}$
	ST/03/2121	5.00×10^{8}	68.00	340.00	6.80×10^{-7}	
		5.00×10^{9}	71.50	357.50	7.15×10^{-8}	
		2.50×10^{9}	99.25	496.25	1.99×10^{-7}	$3.17 \pm 1.85 \times 10^{-7}$
	ST/03/2122	2.50×10^{8}	12.50	62.50	2.50×10^{-7}	
		7.50×10^{8}	12.50	62.50	8.33×10^{-8}	
		5.00×10^{8}	14.50	72.50	1.45×10^{-7}	$1.59 \pm 0.49 \times 10^{-7}$
	MSSA	2.50×10^{9}	8.25	41.25	1.65×10^{-8}	
		5.00×10^{9}	8.50	42.50	8.50×10^{-9}	
		5.00×10^{9}	5.25	26.25	5.25×10^{-9}	$1.01 \pm 0.33 \times 10^{-8}$

Cont.

Table 54. Mutation frequencies of clinical linezolid-resistant isolates to fusidic acid at 4 x MIC (cont.).

Patient no.	Isolate	Colony count (cfu/ml)	No. of mutants in 200 µl	No. of mutants in 1 ml	Mutation frequency	Average mutation freq and SD
	E16	2.50×10^{9}	14.25	71.25	2.85×10^{-8}	
		7.50×10^{9}	7.00	35.00	4.67×10^{-8}	
		1.25×10^{10}	5.75	28.75	2.302×10^{-9}	$1.18 \pm 0.84 \times 10^{-8}$
1a	**H045360367**	1.00×10^{9}	31.50	157.50	1.58×10^{-7}	
		7.50×10^{8}	25.50	127.50	1.70×10^{-7}	
		2.50×10^{9}	49.00	245.00	9.80×10^{-8}	$1.42 \pm 0.22 \times 10^{-7}$
1b	**H053540293**	5.00×10^{8}	6.75	33.75	6.75×10^{-8}	
		5.00×10^{9}	3.25	16.25	3.25×10^{-9}	
		2.50×10^{9}	4.50	22.50	9.00×10^{-9}	$2.66 \pm 2.05 \times 10^{-8}$
2a	**H045360368**	1.00×10^{9}	0.25	1.25	1.25×10^{-9}	
		5.00×10^{8}	3.50	17.50	3.50×10^{-8}	
		7.50×10^{8}	8.25	41.25	5.50×10^{-8}	$3.04 \pm 1.57 \times 10^{-8}$
2b	**H053540294**	7.50×10^{9}	5.00	25.00	3.33×10^{-9}	
		2.50×10^{9}	4.00	20.00	8.00×10^{-9}	
		2.50×10^{9}	2.50	16.50	6.60×10^{-9}	$5.98 \pm 1.38 \times 10^{-9}$

Cont.

Table 54. Mutation frequencies of clinical linezolid-resistant isolates to fusidic acid at 4 x MIC (cont.).

Patient no.	Isolate	Colony count (cfu/ml)	No. of mutants in 200 µl	No. of mutants in 1 ml	Mutation frequency	Average mutation freq and SD
3a	H042800236	1.25×10^{9}	185.00	925.00	7.40×10^{-7}	
		1.75×10^{9}	163.00	815.00	4.66×10^{-7}	
		7.50×10^{8}	23.50	117.50	1.57×10^{-7}	$9.24 \pm 3.31 \times 10^{-7}$
3b	H042800257	5.00×10^{8}	220.00	1100.00	2.20×10^{-6}	
		2.50×10^{9}	163.25	816.25	3.27×10^{-7}	
		2.50×10^{9}	23.50	117.50	4.70×10^{-7}	$9.99 \pm 6.02 \times 10^{7}$
3c	H042800258	2.50×10^{8}	173.00	865.00	3.46×10^{-6}	
		2.50×10^{9}	195.00	975.00	3.90×10^{-7}	
		1.50×10^{9}	19.50	97.50	6.50×10^{-7}	$1.50 \pm 0.98 \times 10^{-6}$
4a	H053760467	5.00×10^{9}	7.00	35.00	7.00×10^{-9}	
		7.50×10^{9}	3.75	18.75	2.50×10^{-9}	
		1.75×10^{9}	5.25	26.25	1.50×10^{-8}	$8.17 \pm 3.66 \times 10^{-9}$
4b	H053760468	5.00×10^{9}	4.50	22.50	4.50×10^{-9}	
		1.75×10^{9}	3.25	16.25	9.29×10^{-9}	
		2.50×10^{9}	3.75	18.75	7.50×10^{-9}	$7.10 \pm 1.40 \times 10^{-9}$

Cont.

Table 54. Mutation frequencies of clinical linezolid-resistant isolates to fusidic acid at 4 x MIC (cont.).

Patient no.	Isolate	Colony count (cfu/ml)	No. of mutants in 200 µl	No. of mutants in 1 ml	Mutation frequency	Average mutation freq and SD
5a	Brazilian 1	2.50×10^{9}	18.75	93.75	3.75×10^{-8}	
		7.50×10^{8}	16.00	80.00	1.07×10^{-7}	
		2.50×10^{9}	16.00	80.00	3.20×10^{-8}	$5.87 \pm 2.40 \times 10^{-8}$
5b	Brazilian 2	1.50×10^{9}	20.00	100.00	6.67×10^{-7}	
		2.50×10^{9}	0.50	2.50	1.00×10^{-8}	
		2.50×10^{9}	17.00	85.00	1.00×10^{-9}	$3.39 \pm 3.28 \times 10^{-7}$

Table 55. Pyrosequencing and hybridization results. (See Table 17, Table 19, Table 26, Table 30, Table 36 and section 3.6).

Strain	Mutation	Lin MIC (mg/L)	23S rRNA copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WT copies
MSSA	N/A	4	6	N/A	N/A	N/A
EMRSA-15	N/A	4	5	N/A	N/A	N/A
EMRSA-16	N/A	4	5	N/A	N/A	N/A
1a	G2576T	16	5	43.3	56.7	2:3
1b	N/A	0.5	4	N/A	N/A	N/A
2a	G2576T	32	5	63.3	36.7	3:2
2b	G2576T	16	4	74.6	25.4	3:1
3a	G2576T	16	6	35.3	64.7	2:4
3b	G2576T	32	6	52.3	47.7	3:3
3c	G2576T	32	6	48.5	51.5	3:3
4a	G2576T	16	4	100.0	0.0	4:0
4b	G2576T	16	4	100.0	0.0	4:0
5a	N/A	2	5	N/A	N/A	N/A
5b	G2576T	32	5	38.4	61.6	2:3
RN4220	N/A	4	6	N/A	N/A	N/A
RN-M1	G2447T	16	6	N/A	N/A	N/A
RN-M2	G2447T	8	6	N/A	N/A	N/A

Cont.

Table 55. Pyrosequencing and hybridization results (cont.).

Strain	Mutation	Lin MIC (mg/L)	23S rRNA copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WT copies
RN-M3	G2447T	16	6	N/A	N/A	N/A
RN-M4	G2447T	16	5	N/A	N/A	N/A
RN-M5	G2447T	16	6	N/A	N/A	N/A
RN-M6	G2447T	16	6	N/A	N/A	N/A
RN4220ΔmutS	N/A	4	6	N/A	N/A	N/A
RN4220Δ*mutS*-M7	G2576T	16	6	33.3	66.7	2:4
RN4220Δ*mutS*-M8	G2576T	16	6	31.9	68.1	2:4
RN4220Δ*mutS*-M9	G2576T	16	6	45.0	55.0	3:3
RN4220Δ*mutS*-M9a	G2576T	>256	6	63.8	36.2	4:2
	A2503G			32.3	67.7	2:4
RN4220Δ*mutS*-M10	G2576T	16	6	48.9	51.1	3:3
ST/03/2121	N/A	4	5	N/A	N/A	N/A
ST/03/2121-M11	T2504C	16	8	38.0	62.0	3:5
ST/03/2121-M12	G2447T	16	6	N/A	N/A	N/A
ST/03/2121-M13	G2447T	16	5	N/A	N/A	N/A
ST/03/2121-M14	G2447T	16	6	N/A	N/A	N/A
ST/03/2121-M15	G2447T	16	4	N/A	N/A	N/A

Cont.

Table 55. Pyrosequencing and hybridization results (cont.).

Strain	Mutation	Lin MIC (mg/L)	23S rRNA copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WT copies
ST/03/2121-M16	Unknown mutation	8	5	N/A	N/A	N/A
ST/03/2121-M17	Unknown mutation	16	7	N/A	N/A	N/A
RN4220	N/A	4	6	N/A	N/A	N/A
M1	G2576T	16	6	51.5	48.5	3:3
M2	G2576T	64	6	64.8	35.2	4:2
M3	G2576T	16	6	46.6	53.4	3:3
M34	G2447T	16	6	N/A	N/A	N/A
RN4220Δ*mutS*	N/A	4	6	N/A	N/A	N/A
M4	G2576T	16	8	46.0	54.0	4:4
M5	G2576T	32	6	54.7	45.3	3:3
M6	G2576T	16	5	41.2	58.8	2:3
M35	G2447T	16	6	N/A	N/A	N/A
M7	T2504C	8	6	36.8	63.2	2:4
M8	T2504C	8	6	12.5	87.5	1:5
M9	G2576T	8	5	37.2	62.8	2:3
M36	N/A	4	6	N/A	N/A	N/A
7499	N/A	4	5	N/A	N/A	N/A
M10	T2504C	16	6	48.3	51.7	3:3

Cont.

Table 55. Pyrosequencing and hybridization results (cont.).

Strain	Mutation	Lin MIC (mg/L)	23S rRNA copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WT copies
M11	T2504C	16	6	32.9	67.5	2:4
M12	T2504C	16	7	42.3	57.7	3:4
M13	T2504C	16	7	30.0	70.0	2:5
M37	N/A	4	5	N/A	N/A	N/A
7500	N/A	4	5	N/A	N/A	N/A
M14	N/A	4	8	N/A	N/A	N/A
M15	Unknown mutation	8	6	N/A	N/A	N/A
M16	G2447T	64	6	N/A	N/A	N/A
M17	G2576T	32	7	58.6	41.4	4:3
M38	G2576T	32	5	58.6	41.4	3:2
M18	N/A	4	6	N/A	N/A	N/A
M19	Unknown mutation	8	6	NA	NA	NA
M20	N/A	4	6	N/A	N/A	N/A
M21	N/A	4	9	N/A	N/A	N/A
M39	Unknown mutation	8	5	N/A	N/A	N/A
7717	N/A	4	5	N/A	N/A	N/A
M22	G2576T	8	6	14.8	85.2	1:5
M23	G2576T	32	9	45.4	54.0	4:5

Cont.

Table 55. Pyrosequencing and hybridization results (cont.).

Strain	Mutation	Lin MIC (mg/L)	23S rRNA copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WT copies
M24	N/A	4	6	N/A	N/A	N/A
M25	N/A	4	6	N/A	N/A	N/A
M40	G2576T	32	6	45.4	54.6	3:3
7501	N/A	4	5	N/A	N/A	N/A
M26	N/A	4	6	N/A	N/A	N/A
M27	N/A	4	5	N/A	N/A	N/A
M28	N/A	4	5	N/A	N/A	N/A
M29	N/A	4	5	N/A	N/A	N/A
M41	N/A	4	5	N/A	N/A	N/A
M30	A2503G	8	6	14.4	85.6	1:5
M31	N/A	4	4	N/A	N/A	N/A
M32	N/A	4	5	N/A	N/A	N/A
M33	N/A	4	6	N/A	N/A	N/A
M42	N/A	4	5	N/A	N/A	N/A
RN4220	N/A	4	6	N/A	N/A	N/A
RN4220 A	T2500A	8	6	17.1	82.9	1:5
RN4220 B	N/A	4	6	N/A	N/A	N/A
RN4220 C	N/A	4	6	N/A	N/A	N/A

Cont.

Table 55. Pyrosequencing and hybridization results (cont.).

Strain	Mutation	Lin MIC (mg/L)	23S rRNA copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WT copies
RN4220 D	N/A	4	5	N/A	N/A	N/A
RN4220 E	G2505A	16	6	14.4	85.6	1:5
RN4220Δ*mutS*	N/A	4	6	N/A	N/A	N/A
RN4220Δ*mutS* A	N/A	2	6	N/A	N/A	N/A
RN4220Δ*mutS* B	N/A	4	6	N/A	N/A	N/A
RN4220Δ*mutS* C	T2500A	8	6	16.7	83.3	1:5
RN4220Δ*mutS* D	N/A	2	6	N/A	N/A	N/A
RN4220Δ*mutS* E	N/A	4	6	N/A	N/A	N/A
ST/03/2121	N/A	4	5	N/A	N/A	N/A
ST/03/2121 A	N/A	4	6	N/A	N/A	N/A
ST/03/2121 B	N/A	4	6	N/A	N/A	N/A
ST/03/2121 C	N/A	2	5	N/A	N/A	N/A
ST/03/2121 D	N/A	4	6	N/A	N/A	N/A
ST/03/2121 E	N/A	4	5	N/A	N/A	N/A
ST/03/2122	N/A	1	5	N/A	N/A	N/A
ST/03/2122 A	N/A	1	6	N/A	N/A	N/A
ST/03/2122 B	N/A	2	6	N/A	N/A	N/A

Table 55. Pyrosequencing and hybridization results (cont.).

Strain	Mutation	Lin MIC (mg/L)	23S rRNA copy no.	Av. % mutated copies	Av. % WT copies	No. mutated : No. of WT copies
ST/03/2122 C	N/A	1	6	N/A	N/A	N/A
ST/03/2122 D	N/A	4	6	N/A	N/A	N/A
ST/03/2122 E	N/A	1	6	N/A	N/A	N/A

Table 56. Mutation frequencies of clinical teicoplanin-resistant isolates to rifampicin (4 x MIC). (See Table 38 for a summary of results)

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
RN4220	7.5×10^9	14.00	70.00	9.33×10^{-9}	
	2.50×10^9	9.00	45.00	1.80×10^{-8}	
	2.50×10^9	8.00	40.00	1.60×10^{-8}	$1.44 \pm 0.26 \times 10^{-8}$
RN4220Δ*mutS*	1.00×10^8	133.25	666.25	6.66×10^{-6}	
	2.50×10^9	175.00	875.00	3.50×10^{-7}	
	2.50×10^9	75.00	375.00	1.50×10^{-7}	$2.39 \pm 2.14 \times 10^{-6}$
ST/03/2121	5.00×10^9	15.00	75.00	1.50×10^{-8}	
	2.50×10^9	17.00	85.00	3.40×10^{-8}	
	5.00×10^9	18.25	91.25	1.83×10^{-8}	$2.24 \pm 0.59 \times 10^{-8}$
MSSA	2.50×10^9	10.00	50.00	2.00×10^{-8}	
	5.00×10^9	11.50	57.50	1.15×10^{-8}	
	5.00×10^9	9.00	45.00	9.00×10^{-9}	$1.35 \pm 0.33 \times 10^{-8}$
EMRSA-15	2.75×10^8	9.50	47.50	1.72×10^{-7}	
	5.00×10^9	11.00	55.00	1.10×10^{-8}	
	2.50×10^9	10.50	52.50	2.10×10^{-8}	$6.80 \pm 5.21 \times 10^{-8}$

Cont.

Table 56. Mutation frequencies of clinical teicoplanin-resistant isolates to rifampicin (4 x MIC)(cont.).

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
EMRSA-16	5.50×10^{9}	8.50	42.50	7.72×10^{-9}	
	2.50×10^{9}	2.50	12.50	5.00×10^{-9}	
	2.00×10^{9}	10.50	52.50	2.63×10^{-8}	$1.30 \pm 0.67 \times 10^{-8}$
H034840069	7.50×10^{8}	23.00	115.00	1.53×10^{-7}	
	2.50×10^{9}	21.25	106.25	4.25×10^{-8}	
	1.75×10^{9}	22.00	110.00	6.29×10^{-8}	$8.62 \pm 3.41 \times 10^{-8}$
H035220342	2.50×10^{9}	15.50	77.50	3.10×10^{-8}	
	5.00×10^{9}	10.75	53.75	1.08×10^{-8}	
	5.00×10^{9}	13.00	65.00	1.30×10^{-8}	$1.83 \pm 0.64 \times 10^{-8}$
H041340156	7.50×10^{9}	4.50	22.50	3.00×10^{-9}	
	2.00×10^{9}	17.50	87.50	4.38×10^{-8}	
	2.50×10^{9}	8.25	41.25	1.65×10^{-8}	$2.11 \pm 1.20 \times 10^{-8}$
H041560345	2.50×10^{9}	39.75	198.75	7.95×10^{-8}	
	2.50×10^{9}	3.25	16.25	6.50×10^{-9}	
	7.50×10^{8}	6.25	31.25	4.17×10^{-8}	$4.26 \pm 2.11 \times 10^{-8}$
H041560348	2.50×10^{9}	8.00	40.00	1.60×10^{-8}	
	1.75×10^{9}	14.50	72.50	4.14×10^{-8}	
	2.50×10^{9}	16.00	80.00	3.20×10^{-8}	$2.98 \pm 0.74 \times 10^{-8}$

Cont.

Table 56. Mutation frequencies of clinical teicoplanin-resistant isolates to rifampicin (4 x MIC) (cont.).

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
H042240308	2.50×10^{9}	5.00	25.00	1.00×10^{-8}	
	1.00×10^{10}	3.50	17.50	1.75×10^{-9}	
	1.00×10^{10}	1.50	7.50	7.50×10^{-10}	$4.17 \pm 2.93 \times 10^{-9}$
H043100413	2.50×10^{9}	12.00	60.00	2.40×10^{-8}	
	2.25×10^{9}	5.25	26.25	1.17×10^{-8}	
	3.00×10^{9}	4.25	21.25	7.08×10^{-9}	$1.43 \pm 0.50 \times 10^{-8}$
H043740188	7.50×10^{9}	14.25	71.25	9.50×10^{-9}	
	2.50×10^{9}	11.25	56.25	2.25×10^{-8}	
	7.50×10^{9}	12.25	61.25	8.17×10^{-9}	$1.34 \pm 0.46 \times 10^{-8}$

Table 57. Mutation frequencies of clinical teicoplanin-resistant isolates to fusidic acid (4 x MIC). (See Table 38 for a summary of results)

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
RN4220	7.50×10^{9}	47.50	237.50	3.17×10^{-8}	
	7.50×10^{8}	48.75	243.75	3.25×10^{-8}	
	2.50×10^{9}	43.50	217.50	8.70×10^{-8}	$5.04 \pm 1.83 \times 10^{-8}$
RN4220Δ*mutS*	1.50×10^{9}	692.00	3460.00	2.30×10^{-6}	
	1.00×10^{9}	202.00	1010.00	1.01×10^{-6}	
	1.50×10^{9}	405.00	2025.00	1.35×10^{-6}	$1.55 \pm 0.39 \times 10^{-6}$
ST/03/2121	2.50×10^{8}	36.00	180.00	7.20×10^{-7}	
	2.50×10^{9}	90.50	452.50	1.81×10^{-7}	
	2.50×10^{9}	42.50	212.50	8.5×10^{-8}	$3.29 \pm 1.98 \times 10^{-7}$
ST/03/2122	1.50×10^{8}	9.50	47.50	3.16×10^{-7}	
	1.00×10^{9}	14.50	72.50	7.25×10^{-8}	
	5.00×10^{8}	29.00	145.00	2.90×10^{-7}	$2.26 \pm 0.77 \times 10^{-7}$
MSSA	1.50×10^{9}	4.50	22.50	1.50×10^{-8}	
	1.50×10^{9}	6.50	32.50	2.17×10^{-8}	
	2.50×10^{9}	3.00	15.00	6.00×10^{-9}	$1.42 \pm 0.46 \times 10^{-8}$

Cont.

Table 57. Mutation frequencies of clinical teicoplanin-resistant isolates to fusidic acid (4 x MIC) (cont.).

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
E15	5.00×10^{9}	<0.25	<1.25	$<2.50 \times 10^{-10}$	
	2.50×10^{9}	<0.25	<1.25	$<5.00 \times 10^{-10}$	
	2.75×10^{8}	<0.25	<1.25	$<4.55 \times 10^{-9}$	$<4.01 \times 10^{-10}$
E16	1.50×10^{9}	10.50	52.50	3.50×10^{-8}	
	2.50×10^{9}	14.25	71.25	2.85×10^{-8}	
	1.50×10^{9}	15.75	78.75	5.25×10^{-8}	$3.87 \pm 0.72 \times 10^{-8}$
H034840069	7.5×10^{8}	123.50	617.50	8.23×10^{-7}	
	2.5×10^{9}	377.00	1885.00	7.54×10^{-7}	
	1.75×10^{9}	73.00	365.00	2.09×10^{-7}	$5.95 \pm 1.94 \times 10^{-7}$
H035220342	2.50×10^{9}	25.25	126.25	2.09×10^{-8}	
	5.00×10^{9}	22.50	112.50	2.09×10^{-8}	
	5.00×10^{9}	24.25	121.25	2.09×10^{-8}	$3.24 \pm 0.91 \times 10^{-8}$
H041340156	7.50×10^{9}	6.25	31.25	4.17×10^{-9}	
	2.00×10^{9}	7.25	36.25	1.81×10^{-8}	
	2.50×10^{9}	2.00	10.00	4.00×10^{-8}	$8.76 \pm 4.86 \times 10^{-9}$
H041560345	2.50×10^{9}	0.25	1.25	$<5.00 \times 10^{-10}$	
	2.50×10^{9}	0.25	1.25	5.00×10^{-10}	
	7.50×10^{8}	0.25	1.25	$<1.67 \times 10^{-9}$	5.00×10^{-10}

Cont.

Table 57. Mutation frequencies of clinical teicoplanin-resistant isolates to fusidic acid (4 x MIC) (cont.).

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
H041560346	2.50×10^{8}	3.75	18.75	7.50×10^{-8}	
	1.00×10^{9}	2.00	10.00	1.00×10^{-8}	
	1.00×10^{9}	3.25	16.25	1.63×10^{-8}	$3.38 \pm 2.07 \times 10^{-8}$
H041560348	2.50×10^{9}	0.50	2.50	1.00×10^{-9}	
	1.75×10^{9}	0.50	2.50	1.43×10^{-9}	
	2.50×10^{9}	0.25	1.25	1.63×10^{-10}	$9.76 \pm 2.68 \times 10^{-10}$
H043100413	2.50×10^{9}	2.75	13.75	5.50×10^{-9}	
	2.25×10^{9}	0.25	1.25	5.56×10^{-10}	
	3.00×10^{9}	0.75	3.75	1.25×10^{-9}	$2.44 \pm 1.55 \times 10^{-9}$
H043740188	7.50×10^{9}	4.50	22.50	3.00×10^{-9}	
	2.50×10^{9}	3.75	18.75	7.50×10^{-9}	
	7.50×10^{9}	3.25	16.25	2.17×10^{-9}	$4.22 \pm 1.66 \times 10^{-9}$
H044640520	2.50×10^{9}	1.00	5.00	2.00×10^{-9}	
	5.00×10^{9}	1.50	7.50	1.50×10^{-9}	
	2.75×10^{9}	1.50	7.50	2.73×10^{-9}	$2.08 \pm 0.04 \times 10^{-9}$
H044640521	7.50×10^{9}	1.75	8.75	1.17×10^{-9}	
	5.00×10^{9}	6.00	30.00	6.00×10^{-9}	
	2.50×10^{9}	4.25	21.25	8.50×10^{-9}	$5.22 \pm 2.15 \times 10^{-9}$

Cont.

Table 57. Mutation frequencies of clinical teicoplanin-resistant isolates to fusidic acid (4 x MIC) (cont.).

Isolate	Colony count (cfu/ml)	Av. No. of mutants in 200 µl	Av. No. of mutants in 1 ml	Mutation frequency	Av. mutation freq and SD
H044920446	2.50×10^{9}	3.75	18.75	7.50×10^{-9}	
	2.50×10^{9}	3.00	15.00	6.50×10^{-9}	
	2.75×10^{9}	4.50	22.50	8.18×10^{-9}	$7.33 \pm 0.64 \times 10^{-9}$
H045000304	2.50×10^{9}	0.50	2.50	1.00×10^{-9}	
	2.50×10^{9}	2.50	12.50	5.00×10^{-9}	
	2.00×10^{9}	1.25	6.25	3.13×10^{-9}	$3.04 \pm 1.16 \times 10^{-9}$

Publications arising from this work

North, S. E., M.J.Ellington, A.P.Johnson, D.M.Livermore, and N.Woodford. 2005. *In-vitro* selection of linezolid resistance in hypermutable and wild-type *Staphylococcus aureus.* Clin.Microbiol.Infect. **11:**(Suppl 2)299

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